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CLEVELAND, OHIO	
University Hospitals	October 13, 1953 November 16, 1953 December 14, 1953
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ILLINOIS	
Michael Reese Hospital	December 8, 1953
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Tulane University	November 20, 1953

Sodium and Potassium Exchanges During Dialysis of Bilaterally Nephrectomized Dogs.* (20767)

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Dogs subjected to bilateral nephrectomy and maintained by dialysis develop a series of abnormalities among which have been emphasized hypertension(1,2), cardiovascular lesions(3,4), hemolytic anemia(5), and a negative nitrogen balance(6). The present communication considers the relationship between external sodium and potassium balance and changes in the content of sodium and potassium in the "extracellular fluid" as measured by the distribution of radiosulfate in association with dialysis following bilateral nephrectomy of the dog.

Methods. All methods used have been identified in another presentation(7). The nephrectomies were performed one week apart and peritoneal irrigation was begun after the second nephrectomy. The control mean arterial pressures, which varied between 110 and 120 mm Hg, and the body weights were obtained by averaging the daily figures obtained during the week before the second nephrectomy. Dog Nos. 1 to 15 received a diet containing an average of 77 calories/kg/day and between 1.4 and 4.2 g protein (mainly casein)/kg/day (average 2.7 g/kg/day) given in an average volume of 191 cc/day. Dogs Nos. 16 to 19 were handled in a similar manner except that they received an average of 45 cals/kg/day and protein (casein) in an amount of 1 g/kg/day in an average volume of 140 cc/day. Vomiting was negligible in 7 dogs and diarrhea did not occur. External Na and K balances were determined by the quantities in the diet and the quantities entering and leaving the peritoneal cavity during the daily dialyses. Fluid equilibrium was maintained in most instances during the dialyses.

The radiosulfate (S^{35}) space was obtained

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by a modification of the method of Walser (8). Radioactive S^{35} as sodium sulfate was injected intravenously in doses of 100 μc or less and serum samples were taken at 15, 30, 60, and 120 minutes. Adequate mixing was obtained after 15 minutes; the minute count fell in a straight line with a slight slope when plotted on semilogarithmic paper. The counts were obtained with a flow counter as follows: A sample of undiluted serum (0.1 to 0.2 cc) was allowed to spread on a disc of lens paper (O.D. 2 cm) impregnated with a 5% suspension of aquadag®† and mounted on an aluminum disc. The aquadag aided in the uniform dispersion of serum over the counting surface and minimized self-absorption and back-scattering. The unknown sample was compared with a known sample of normal serum containing diluted radiosulfate treated in a similar manner. The samples were counted until 11 x 64 counts were obtained. The Na, K, and radiosulfate concentrations were expressed in the ECF water by correcting for the plasma protein concentration. The radioactivity was extrapolated back to zero time on semilogarithmic paper while measuring the radiosulfate space. Consideration has been given to the gain or loss of Na or K from or to the outside and the gain or loss of Na and K in the "extracellular fluid" as measured by radioactive sulfate. These two quantities (exogenous balance and extracellular fluid balance) have been considered to indicate the quantities of Na and K either leaving or entering the extracellular space for or from other compartments in the body, presumably cellular. Thus, the sodium gained from the outside and the sodium depleted from the extracellular fluid are considered collectively as the likely quantity of sodium gained by cells, and the potassium lost to the outside and the potassium gained by the extracellular fluid are considered collectively as the likely quantity of K lost by cells.

Results. The results are given in Table I. During the period of study (3 to 7 days) the radioactive sulfate space was either essentially unchanged or significantly lowered. In 16 dogs the total "extracellular" sodium decreased, while in 15 dogs there was either a negligible or definite gain of sodium from the outside. This indicated in 16 dogs an apparent gain in cellular sodium varying between 39 and 226 mEq, in 11 dogs it exceeded 50 mEq. In 8 dogs, which either lost up to 36 mEq of Na to the outside or gained up to 31 mEq, 5 showed apparent cellular gains varying between 21 and 49 mEq. Only 3 dogs revealed changes which might be commensurate with decrements in cellular sodium. The apparent shift in Na away from the ECF bore no constance relationship to the serum Na concentration.

All animals were maintained in a state of negative exogenous K balance, since the diet contained between 2 and 4 mEq of K per day and the input irrigating fluid was devoid of K while upon exit it contained between 3.5 and 7.8 meq/liter. However, there were special aspects of the loss of K to the outside. Sixteen dogs displayed an elevation of the serum K concentration, and of these 11 showed an increment in the total "extracellular" K. Thus, the negative exogenous K balance, mediated via the irrigation, appeared to be potentiated by a tendency toward the passage of K from some compartment, presumably cellular, into the extracellular fluid. By the methods used all animals displayed an apparent decrement in the cellular K.

Comment. The gain in exogenous Na plus a decrement of Na in the ECF as measured suggest the shift of Na into cells, while a loss of K to the outside and an increment of K in the ECF as measured suggest a shift of K out of cells. Even in certain animals displaying no exogenous sodium gain, there was a tendency for Na to be deviated out of the ECF.

No constant quantitative relationship was detected between the shifts of Na and K. Thus, certain animals accruing larger quantities of Na, lost lesser equivalent quantities of K, while some animals that lost greater quantities of K gained a lesser equivalent amount of Na. These discrepancies, which suggest that the entire study indicates a trend only, may result partly from internal factors. That

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† Colloidol carbon in water, manufactured by Acheson Colloids Corp., Port Huron, Mich.

TABLE I. Data from 19 Dogs as Discussed in Text.

Wt, kg	No. days	Change ECF (S^{35}) space, cc	Change serum water Na, mEq/L	Change serum water K, mEq/L	Change ECF Na, mEq	Exogenous Na balance, mEq	Apparent cellular Na change, mEq	Change ECF K, mEq	Exogenous K balance, mEq	Apparent cellular K change, mEq	Change mean BP, mm Hg
16.5	5	— 55	—16	+2.3	—43	+ 6	+ 49	+4.0	—26	—30	+45
11.0	5	— 82	— 9	+2.4	—27	+ 16	+ 43	+3.7	—59	—63	+40
15.5	4	—164	—24	+1.4	—98	+103	+201	+3.6	—37	—40	+32
15.5	4	—295	—16	+1.5	—93	+ 46	+139	+2.4	—47	—49	+50
13.4	7	+ 92	+13	— .3	+44	+179	+135	— .1	—58	—58	+20
12.3	5	—437	— 1	— .8	—69	+157	+226	—4.7	—29	—24	+55
10.9	4	— 91	+ 3	+2.5	— 8	+ 31	+ 39	+3.6	—29	—33	+40
10.3	4	+ 29	+ 7	+1.3	+15	— 34	— 49	+2.3	—33	—35	+50
10.3	4	—261	— 3	+ .4	—44	— 23	+ 21	—1.0	—29	—28	+40
13.2	3	—510	+ 4	+ .7	—74	+ 78	+152	—1.4	—17	—16	+30
16.4	3	—642	+ 5	+ .8	—81	+ 61	+142	—2.2	—19	—21	+50
10.7	3	—125	+ 6	+2.1	—10	— 29	— 19	+2.5	—22	—24	+35
9.8	3	—277	+ 7	+2.4	—28	+ 11	+ 39	+2.3	—21	—23	+60
11.8	3	—253	+11	— .1	—19	— 36	— 17	—1.3	—18	—17	+35
8.4	5	—523	+ 4	+1.1	—76	+ 37	+113	—1.0	—46	—45	+30
9.9	4	— 93	+ 2	+1.6	—11	+ 73	+ 84	+2.0	—27	—29	+25
8.5	4	+ 32	+ 6	+1.4	+11	+ 67	+ 56	+1.4	—22	—23	+25
7.5	3	—347	+ 1	+1.9	—50	+ 95	+145	+1.0	—22	—23	+37
12.1	3	—731	+ 4	+1.2	—105	+ 76	+181	—1.1	—13	—12	+50

internal factors are involved in the movement of K is indicated by the relationship of the loss of nitrogen and K in the outflow irrigating fluid, as discussed elsewhere(7). By nitrogen balance studies it has been shown(7) that the quantity of nitrogen removed by the irrigating fluid, which apparently is derived from cellular breakdown, far exceeds the expected quantity of K derived from the tissues. This indicates that although K passes into the ECF some of it is deviated or combined elsewhere in the body.

Hypertension was sustained by all 19 dogs while cardiovascular lesions were observed in 90% of the animals studied morphologically. Whether the electrolytic changes were related to the heightened arterial pressure could not be ascertained by these studies.

Conclusions. 1. Bilaterally nephrectomized dogs subjected to dialysis, under the conditions described, tend to gain Na from the dialyzing fluid and lose K into the fluid while at the same time measurements of the ECF volume by radiosulfate suggest a tendency toward a decrement of total ECF Na and an increment of total ECF K. 2. These findings are in keeping with a tendency toward a gain of cellular Na and a loss of cellular K. 3. A movement of Na into cells may occur even in the absence of a positive exogenous Na balance.

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1. Grollman, A., Muirhead, E. E., and Vanatta, Jr., *Am. J. Physiol.*, 1949, v157, 21.

2. Leonards, J. R., and Heisler, C. R., *Am. J. Physiol.*, 1951, v167, 553.

3. Muirhead, E. E., Grollman, A., and Vanatta, Jr., *Arch. Path.*, 1949, v48, 234.

4. Orbison, J. R., Christian, C. L., and Peters, E., *Arch. Path.*, 1952, v54, 185.

5. Muirhead, E. E., Jones, F., Stirman, J. A., and Lesch, W., *Am. J. Physiol.*, 1953, v173, 371.

6. ———, *Surg. Gyn. and Obst.*, 1953, v97, 189.

7. Muirhead, E. E., Jones, F., and Graham, P., *Circ. Res.*, 1953, v1, 439.

8. Walser, M., PROC. SOC. EXP. BIOL. AND. MED., 1952, v79, 372.

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Identification of the Substance Active in Sheep Cell Agglutination Test for Rheumatoid Arthritis. (20768)

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Rose *et al.*(1) proposed a sheep erythrocyte agglutination test for rheumatoid arthritis in which the serum of arthritic patients, previously treated to remove a non-specific agglutinating factor, specifically agglutinates sheep red cells sensitized with sheep cell antibody. Freund and Stulberg(2), and others (3,4) have introduced modifications to increase the sensitivity of this test and have made studies of its reliability. The electrophoretic pattern of plasma from arthritic patients shows the presence of a large amount of mucoprotein(5,6) and gamma globulin (7-9), and a characteristic peak in the descending boundary pattern(10). The factor responsible for the agglutination was reported to be a globulin(1). We have attempted to concentrate the plasma factor responsible for this test and to determine its chemical nature.

Methods and materials. The sheep cell agglutination test(1) as modified by others (3,4) was further modified and used as follows: A. Preparation of Red Blood Cells. Sheep red blood cells, preserved with Alsever's solution, were washed 3 times with physiological saline, and used as packed cells, and as 1% and 0.5% suspensions in saline. B. Preparation of Sensitized Red Blood Cells. Commercial anti-sheep red cell hemolysin was diluted in serial 2-fold dilution, and 0.5 ml of each dilution was mixed with 0.5 ml of a 0.5% suspension of sheep cells. The mixtures were incubated for one hour at 37°C, allowed to stand overnight at 4°C, and then examined for agglutination of the red cells. The agglutinating titer of hemolysin was designated as the highest dilution which produced agglutination. Hemolysin solution containing ½ agglutinating titer was incubated with an equal volume of a 1% suspension of sheep red cells for 30 minutes at room temperature. The sheep cells were now designated "sensitized". C. Preparation of Patients' Plasma and Observations of Hemagglutination Titers. The normal agglutinating component in plasma was removed by heating each plasma specimen at 56°C for 30 minutes and then adsorbing the factor on sheep red cells added in the ratio of one volume of packed cells to 4 volumes of plasma. After 40 minutes the red cells were removed by centrifugation and the plasma treated a second time with sheep cells. The plasma was then diluted in serial 2-fold dilution to a final concentration of 1/5120, and 0.5 ml of sensitized sheep cells added to 0.5 ml of each dilution. Control tubes were prepared by diluting plasma 1/10, 1/20, and 1/40 and treating 0.5 ml of each dilution with 0.5 ml of a 0.5% suspension of unsensitized red cells. All tubes were incubated at 37°C for one hour, allowed to stand overnight at 4°C, and the degree of agglutination read as 1+, 2+, 3+ or 4+. The number of agglutination units present in 100 ml of undiluted plasma was defined as the number of dilutions required to give a 1+ agglutination test under the conditions of the test. The electrophoretic patterns were obtained (11,12) with the Longsworth modification of the Tiselius instrument using an acetate buffer pH 4.5 for mucoprotein, and a barbital buffer pH 8.5 for the other plasma proteins.

Results. A comparison of the amount of the agglutinating factor with the amount of mucoprotein in the plasma of normal and of diseased patients indicates that the agglutinating factor is not mucoprotein (Table I). High levels of agglutinating factor were always associated with high levels of mucoprotein. However, low levels of agglutinating factor, particularly in cancer, were also associated with high levels of mucoprotein. In addition, it was found that mucoprotein prepared from arthritic blood having a high agglutination titer, by the method of Mehl *et al.*(13) caused no agglutination in the absence of other serum proteins. In order to concentrate the agglutination factor, pooled

TABLE I. Relation of Amount of Mucoprotein to Amount of Agglutinin in Plasma.

Patient	Diagnosis	Total protein, g/100 ml	Mucoprotein, relative amt	Agglutination, units*
R	Normal	7.28	1	20
W		6.89	1	10
S		7.31	1	20
N	R.A.†	8.10	3	5120
Wo		9.59	3	80
D		8.88	3	5120
O		8.00	2	1280
P		9.53	1.5	640
F		7.60	2.5	80
C	Cancer	6.03	3	20
C		6.99	2	40
Sh	Still's disease	7.53	4	160

* For definition, see text.

† R.A.—Rheumatoid arthritis.

samples of arthritic plasma were fractionated by the Cohn ethanol method(14), by the use of sodium sulfate(15) and by dialysis and precipitation at low ionic strength. The addition of alcohol to arthritic sera caused the loss of all agglutinating activity. This was unexpected in view of a report by others(16) that lipid extraction with an alcohol-ether mixture does not destroy this factor.

In the fractionation of the serum of arthritic patients by the addition of sodium sulfate (Table II), the precipitate obtained at a concentration of 9% contained only a small fraction of the agglutination factor present in the plasma. Further addition of sodium sulfate to a concentration of 14% gave an additional small amount of protein precipitate which contained the highest concentration of agglutination factor, per gram of protein, of any fraction obtained. Further addition of sodium sulfate to a concentration of 18.6% caused the precipitation of protein possessing little activity; the supernatant solution was almost devoid of activity. The activity present in the original arthritic pool was recovered quantitatively in the various fractions within the rather wide limits of error of the method of determination. The electrophoretic examination of the active precipitate after extensive washing with the precipitating salt solution revealed the presence of alpha, beta, and gamma globulin. Rose *et al.*(1) reported that the activity was present in electrophoretically separated beta-gamma globulin fraction.

For the separation of the agglutination factor by dialysis, 100 ml of pooled plasma was dialyzed in Visking tubing against successive 4000 ml volumes of phosphate buffer of pH 6.8 and ionic strength varying from 0.1 to 0.01 (Table III). The precipitate formed after each period of dialysis was removed by centrifugation and the serum then replaced in the Visking tubing and dialyzed against a buffer of lower ionic strength. Dialysis was carried out at 4°C. For each dialysis 2 days were required for the composition of the 2 solutions to reach equilibrium. High concentrations of the agglutination factor were obtained in the fractions precipitated at ionic strength 0.1 and at ionic strength 0.06 to 0.04. This might suggest that the substance is not a single factor. The precipitation of the agglutination factor at ionic strength 0.1, however, may well be attributed to adsorption on fibrinogen, which is a principal constituent of this fraction, rather than to precipitation as the free factor. The protein present in this fraction had an electrophoretic mobility lower

TABLE II. Sodium Sulfate Fractionation of Plasma of Arthritic Patients.

Fraction*	Protein, g/100 ml plasma	Agglutination, units†	Agglutination, units/g protein
1. Whole plasma	6.10	640	110
2. Defibrinated plasma	5.70	1230	220
3. Precipitate from 9% Na_2SO_4	.30	20	70
4. Supernatant from (3)	5.10	640	130
5. Precipitate from 9-14% Na_2SO_4	.36	640	1600
6. Precipitate from 14-18.6% Na_2SO_4	1.72	160	90
7. Supernatant from (6)	3.00	20	6

* Precipitates were formed by successive addition of sodium sulfate to indicated concentrations. Concentrations are expressed as g/100 ml of final solution.

† For definition, see text.

TABLE III. Fractionation of Arthritic Plasma by Reduction of Ionic Strength.

Fraction*	Protein,† g/100 ml	Mobilities of electrophoretic peaks, $cm^2 volt^{-1} sec^{-1}$ $\times 10^{-5}$	Agglutination, units†	Agglutination, units/g protein
1. Whole plasma	8.0	6.1 5.1 4.0 2.9 2.1 1.2	640	80
2. Precipitate at ionic strength .10	.08	2.0	40	500
3. ” ” ” ” .08	.12	4.8 2.4 1.1	20	166
4. ” ” ” ” .06	.40	6.1 3.0 2.2	160	400
5. ” ” ” ” .04	.21	3.2 6.3	160	800
6. ” ” ” ” .02	.54	4.0 3.2 2.7	320	590
7. ” ” ” ” .01	.50	6.3 3.9 3.1 2.5 1.0	40	80

* Precipitates were formed by successive dialyses of plasma of arthritic patients against phosphate buffer solutions of pH 6.8 and varying ionic strengths.

† As g of protein or agglutination units in 100 ml of plasma or the precipitate from 100 ml of plasma.

than that of fibrinogen (2.0 compared with 2.4 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}), thus suggesting that the fibrinogen might be adsorbed to another protein. In addition, it was observed that in those experiments in which clotting occurred during dialysis, a considerable portion of the agglutination factor was adsorbed on the clot. This adsorbed factor could be removed from the clot only by prolonged washing with physiological saline. When the clot was lysed with fibrinolysin, the agglutination factor was also destroyed.

Those fractions precipitated at ionic strength between 0.08 and 0.02 contained relatively high concentrations of agglutination factor. The electrophoretic patterns of these fractions showed peaks corresponding to those of albumin, alpha and beta globulin, and fibrinogen, 6.0, 4.5, 2.9, and 2.4 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, respectively. However, the only peak appearing in all the fractions exhibiting agglutinating activity is the beta peak ($\mu =$ 2.0-3.2 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}). The agglutination factor appears to be either precipitated as such or to be adsorbed on the beta globulin and then precipitated with this fraction. The pool of arthritic blood plasma exhibited an electrophoretic anomaly in the beta fraction similar to that reported by Reed *et al.*(10). This anomaly was not observed in the electrophoretic patterns of the concentrates.

A further attempt at purification of the agglutination factor consisted of an initial precipitation with sodium sulfate, added in the amount of 18.6 g per 100 ml of final solution, and subsequent fractionation by dialysis and lowering of the salt content. The highest titer of agglutinating factor was again found in the fraction precipitating between 0.06 and 0.04 ionic strength. The activity of the various fractions and the electrophoretic analyses of the precipitates were essentially the same as for the previous experiments.

When the concentrated fractions of agglutination factor were lyophilized, all activity was lost. This was unexpected as whole plasma containing the active fraction may be kept indefinitely when treated in this manner.

Summary. The active principle responsible for sheep cell agglutination test for arthritis was purified and concentrated by salt fractionation and by dialysis against solutions of low ionic strength. The most active fractions were precipitated as plasma was adjusted from a concentration of 9% to a concentration of 14% sodium sulfate, or from ionic strength 0.06 to ionic strength 0.04. The electrophoretic fraction most consistently present in the concentrate was beta globulin. The agglutination factor is adsorbed on fibrin and is inactivated during lyophilization of concentrates and by the addition of alcohol to arthritic plasma.

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1. Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 1.

2. Freund, H. A., and Stulberg, C. S., *J. Mich. Med. Soc.*, 1950, v49, 1084.

3. Pike, R. M., Sulkin, S. E., and Coggeshall, H. C., *J. Immunol.*, 1949, v63, 441.

4. Heller, G., Jacobson, A. S., and Kolodny, M. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 316.

5. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *Fed. Proc.*, 1950, v9, 244.

6. Kelly, V. C., Good, R. A., and McQuarrie, *J. Pediatrics*, 1950, v5, 824.

7. Lovgren, O., *Acta Medica Scandinavica*, Supplementum CLXIII, 1945, 61.

8. Dole, V. P., and Rothbard, S., *J. Clin. Invest.*, 1947, v26, 87.

9. Perlman, G. E., and Kaufman, D., *ibid.*, 1946, v25, 931.

10. Reed, A. F., Pike, R. M., Sulkin, E., and Coggeshall, H. C., *J. Lab. Clin. Med.*, 1951, v37, 264.

11. Mehl, J. W., Golden, F., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 110.

12. Coryell, M. N., Beach, E. F., Robinson, A. R., Macy, I. G., and Mack, H. C., *J. Clin. Invest.*, 1950, v29, 1559.

13. Mehl, J. W., Humphrey, J., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 106.

14. Cohn, E. G., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

15. Majoor, C. L. H., *J. Biol. Chem.*, 1947, v169, 583.

16. Pike, R. M., Sulkin, S. E., and Coggeshall, H. C., *J. Immunol.*, 1949, v63, 447.

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Effects of Cortisone on Blood Cells after Thermal Stress.* (20769)

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In the course of experiments on inflammatory phenomena (to be published) rabbits were subjected to extensive thermal injuries, and data on erythrocytes, leukocytes, and temperature were recorded. It was considered worthwhile to make a parallel series of records on rabbits treated with cortisone. A comparison of these is the basis for this report.

Methods. Male New Zealand whites weighing 1800 g to 2300 g were used. They were given a routine diet of rabbit pellets, greens and water during a period of one to 3 weeks and frequent blood counts were made to establish the norm for each animal. None was used if significant variations in blood count, temperature, or behavior indicated that the animal's health was not normal. Experiments were made on 4 groups of 10 each. The fur over the back, shoulders, sides and hips was sheared closely with clippers. The rabbit was anesthetized deeply with ether and the areas mentioned were dipped for 5 seconds into water at a temperature of 88° to 95°C. Pain was prevented by frequent injections of morphine during the subsequent 24 hours. Blood counts and temperature were taken at intervals ranging from 30 minutes to 7 hours

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* We are indebted to Merck and Co. for generously supplying the Cortone® Acetate.

and again at 24 hours, after which the animal was anesthetized and killed. The scalded areas were estimated at about ⅓ of the total skin surface. One group of 10 rabbits had received cortisone by intramuscular injection as follows: on the day before experimentation, 2 injections of 12.5 mg each, and one injection of the same amount just before the experiment began. The same dosage of cortisone was given to another group of 10 rabbits before thermal injury by freezing. Rabbits were prepared and anesthetized for freezing as described above for scalding. A freezing mixture was made by placing lumps of solidified CO_2 (dry ice) into a basin of 95% alcohol. The back, shoulders, sides and hips of the animal were immersed in this for 60 seconds at temperatures ranging from —50°C to —60°C. Two groups of 10 each were subjected to freezing. Anesthesia, tests and after-treatment were identical to those described for injury by heat.

Results. Erythrocytes. A prompt increase in the count of red cells occurred in every instance. This was evident in some rabbits within 15 minutes after scalding and reached its maximum within one to 6 hours. The average degree of hemoconcentration for each group and the speed of its development are shown graphically in Fig. 1. Fig. 2 portrays the same features after freezing.

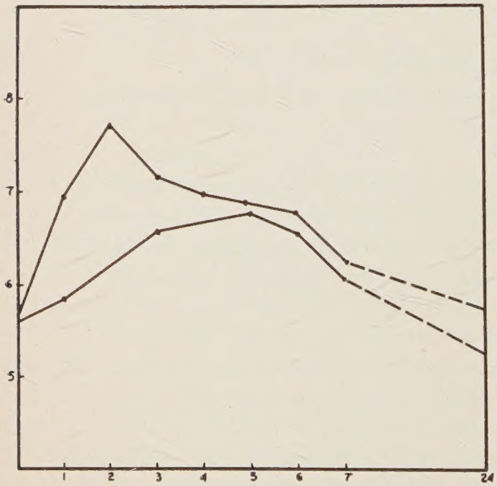

FIG. 1. Hemoconcentration after Stress by Heat. Red cells in millions are shown at the left; elapsed time in hr is shown at the bottom. The upper graph represents untreated animals; the lower graph represents animals treated with cortisone.

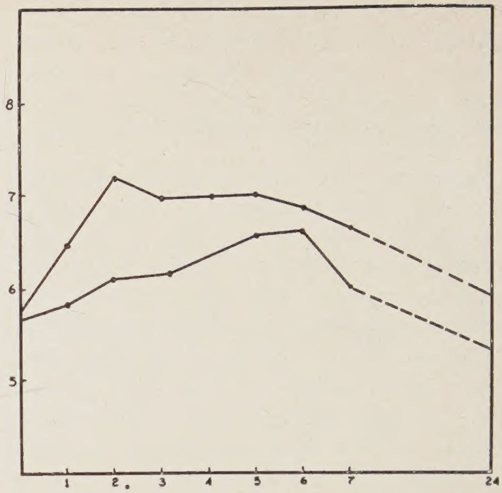

FIG. 2. Hemoconcentration after Stress by Freezing. Details as for Fig. 1.

The curves in Fig. 1 depict the rapid development of hemoconcentration in animals after stress by heat. The average concentration of erythrocytes rose sharply after injury and reached its peak 2 hours later. In contrast, the rise was more gradual in the cortisone-treated animals. The peak was lower and was reached 3 hours later than that of the untreated group. This represents a time lag of 150%. In other words, the development of hemoconcentration was definitely retarded under the influence of cortisone. The variations in hemoconcentration after stress by freezing (Fig. 2) are of the same kind but less in degree than those after injury by heat. Again, the curve in cortisone-treated animals rose more slowly and reached its maximum 3 hours later than that of the untreated group. The fact that these curves are lower than in Fig. 1 may indicate that scalding was a more severe injury than freezing. We have found no reports on the effects either of ACTH or of cortisone upon hemoconcentration during stress of whatever origin.

It is well known that hemoconcentration develops in man after severe burns. Usually this subsides within 2 or 3 days and is succeeded gradually by anemia. Students of capillary physiology have attributed hemoconcentration to endothelial effects. Krogh

(1) and Lewis(2) showed that when epidermal cells are injured by light, heat, trauma, various toxins or chemicals, they release a substance (H-substance—Lewis) which causes dilation and increased permeability of the capillaries locally. This results in hyperemia and edema, due to leakage of plasma into the tissue spaces. Landis(3) stated that any type of injury to endothelium increases the permeability of capillaries to the plasma colloids. Loss of plasma leaves the blood more concentrated. There is evidence(4) that in cases of extensive injury the capillaries in systemic areas are similarly affected, though not everyone supports this belief. Certainly in extensive burns in man, the ensuing hemoconcentration is due largely to local loss of plasma as blister fluid. Burns do not cause blisters nor denuded oozing surfaces on rabbits and other animals, but local edema develops under burned areas. Whether or not superficial burns produce capillary permeability in systemic areas, the present knowledge of capillary physiology supports the explanation that hemoconcentration after burns results from abnormal permeability of endothelium to plasma colloids.

Kendall(5) demonstrated that one important function of adrenal cortical hormones is to govern permeability and to control the movement of water and of electrolytes between the blood and the tissues. This has been substantiated by Selye(6) and others, and it has been shown that Compound E (cortisone) is largely responsible for this effect. Best and Taylor(7) state that the cortical steroids reduce capillary permeability. Ebert(8) and Lurie(9) reported that cortisone has this effect in reactions to injury. Balourdas and Chambers(10) found that cortisone caused a striking reduction of capillary leakage in the exteriorized mesoappendix of rats. We(11) found a definite decrease in the amount of edema formed in experimental inflammation under cortisone treatment. This was attributed to lessened capillary permeability. Whitelaw(12) used ACTH in the treatment of patients suffering extensive burns; he reported that oozing from burned surfaces decreased progressively. Hemoconcentration after burns and early in secondary shock from other causes is accompanied by a decrease in the volume of circulating blood. The evidence cited indicates that this is due to leakage of plasma through abnormally permeable endothelium and that cortisone tends to lessen this effect. Hence cortisone tends to sustain the blood volume and to retard hemoconcentration. We have found no report that cortisone actually increases blood volume except after prolonged use, when signs of Cushing's syndrome develop. Finch *et al.* (13) recorded blood volumes and other items in 20 patients during several courses of cortisone treatment; no significant changes in blood volume were found. We believe that the retarded hemoconcentration in our experiments was due to the effects noted by these authors. We have no evidence whether the lessened permeability caused by cortisone was other than local.

Leukocytes. Cortisone caused a significant reduction in the total count of white cells in the blood. This leukopenia explains the low initial point for the leukocytic curves of cortisone-treated animals in Figs. 3 and 4. In 17 rabbits, differential counts had been made both before and after cortisone was given and prior to thermal injury; in 3, only total white counts had been made. The average white cell count of this group was 6,350.

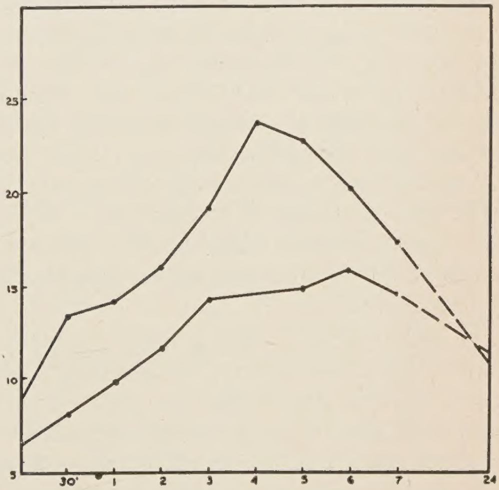

FIG. 3. Leukocytosis after Stress by Heat. Leukocytes in thousands are indicated at the left; elapsed time in hr is shown at the bottom. Untreated and cortisone treated animals are shown on the upper and lower graphs respectively.

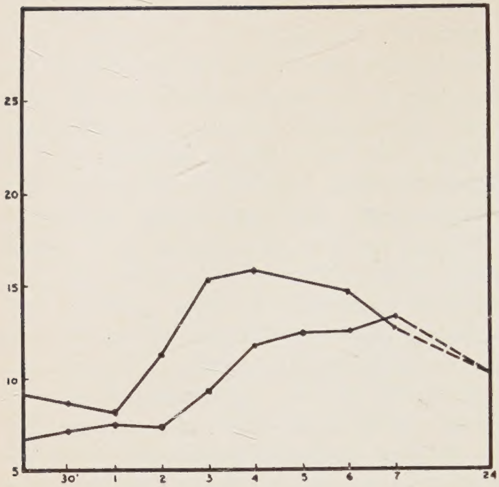

FIG. 4. Leukocytosis after Stress by Freezing. Details as in Fig. 3.

This decline is attributed largely to lymphocytopenia after cortisone treatment. The average percentage of lymphocytes in this group was 58%; after cortisone injections and before injury, the lymphocyte count dropped to 27%—a difference of 31%. Now, if 8,775 is reduced by 30%, it leaves 6,143 which is near to 6,350—the average total white count found after cortisone treatment. The lymphocytopenic effect of cortisone has been observed by other workers (see Dougherty)(14). It is well known that extensive superficial burns in man cause an immediate leukocytosis. We have not found similar reports after exposure to severe cold, though this is certainly a powerful stressing agent. In the experiments here described, white cell counts were made both before and at intervals after thermal stress, both heat and cold, in 10 cortisone-treated and in 10 untreated rabbits. The results are shown graphically in Figs. 3 and 4.

It is seen (Fig. 3) that a sharp rise in the total white count occurred immediately after injury by heat, and reached its peak 4 hours later. In the cortisone-treated animals the rise was more gradual, the peak was not so high and was reached 2 hours later than in the untreated group. Differential counts showed the increase to be in the percentage of polymorphonuclear cells. There was a moderate decline in the percentage of lymphocytes found in the blood of the untreated animals. Before stress by heat, the lymphocytes were 54.7% in this group; 4 hours later it had declined to 42.2%. Probably this has little significance, since the high increase in neutrophiles would result in a lower relative percentage of lymphocytes. Leukocytosis in each group had decreased greatly in 24 hours, but had not subsided completely. No examinations of blood were made during the interval between 7 and 24 hours.

We(15) have shown evidence that substances derived from damaged cells attract leukocytes into areas of local injury, as after burns, freezing or trauma. One of us(16) advanced the explanation that probably the same substances produce a systemic mobilization of leukocytes when released in large amounts by extensive injuries of the same kind. The fact is well attested that extensive burns or trauma are followed promptly by leukocytosis. This occurs too rapidly and subsides too soon to be caused by infection. Our results indicate that cortisone retards the leukocytosis which follows burns.

After freezing, blood counts made at 30 minutes and at 60 minutes showed a *decline* in white cells in the 10 untreated animals (Fig. 4). This was followed by a sharp rise reaching its peak 4 hours after the injury. In the cortisone-treated animals, no significant changes occurred until 2 hours after freezing, and the peak was reached at 7 hours—3 hours later than in the untreated group. Apparently, cells injured by freezing do not so promptly liberate substances which affect the count of leukocytes, as do cells injured by heat.

Summary. 1. Our results indicate that cortisone inhibits and retards the development of hemoconcentration after thermal stress in rabbits. We attribute this to its property of maintaining endothelial impermeability. 2. After injections of cortisone the total white cell count is lowered, due largely to severe lymphocytopenia. This finding agrees with previous reports by others. 3. Thermal stress in rabbits is followed by leukocytosis. This is of less degree and develops more slowly under the influence of cortisone.

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1. Krogh, A., *Anatomy and Physiology of the*

Capillaries. New Haven, Yale University Press, 2nd ed., 1929.

2. Lewis, Thos., *Blood Vessels of the Human Skin and their Responses.* London, Shaw and Sons, 1927.

3. Landis, E. M., (a) Capillary pressure and capillary permeability, *Phys. Rev.,* 1934, v14, 404; (b) *Am. J. M. Sc.,* 1937, v193, 297.

4. Moon, V. H., Hemoconcentration. *Am. J. Clin. Path.,* 1941, v11, 361.

5. Kendall, E. C., *Arch. Path.,* 1941, v32, 474.

6. Selye, H., *Stress,* Montreal, Acta, Inc., 1950.

7. Best, C. H., and Taylor, N. B., *The Physiologic Basis for Medical Practice,* Baltimore, Williams and Wilkins Co., ed. 5, 1950, p313.

8. Ebert, R. H., *J. Clin. Invest.,* 1951, v30, 636.

9. Lurie, M. B., Zappasodi, P., Dannenberg, A. M., Jr., and Lynch, E. C., Abstr., 47th Ann. Meet., Nat. Tuberc. Assn., May 14, 1951, p4-5.

10. Balourdas, T., and Chambers, R., *Fed. Proc.,* 1952, v11, 8.

11. Moon, V. H., and Tershakovec, Geo. A., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 63.

12. Whitelaw, M. J., Proc. 2nd Clin. ACTH Conf., Philadelphia, Blakiston, 1951, p310.

13. Finch, S. C., Crockett, C. L., Ross, J. F., and Bayles, T. B., *Blood,* 1951, v6, 1034.

14. Dougherty, T. F., Effect of Hormones on Lymphoid Tissues, *Phys. Rev.,* 1952, v32, 379.

15. Moon, V. H., and Tershakovec, G., *A. M. A. Arch. Path.,* 1951, v52, 369.

16. Moon, V. H., *Shock and Related Capillary Phenomena,* New York, Oxford Press, 1938, p76.

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Comparison of Hypotensive Action of Sodium Azide in Normotensive and Hypertensive Patients.* (20770)

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In the course of investigations on the effects of various inhibitors on human cancer patients (1), it was noted in cases with co-existent hypertension that the acute administration of sodium azide caused a temporary lowering of the blood pressure toward normotensive levels. In contrast, comparable doses of azide produced no appreciable change in the blood pressure of normotensive individuals. These incidental observations led to a more extended study of the hypotensive effect of sodium azide in both man and experimental animals. A review of the literature indicated that Graham(2) had made an intensive study of the pharmacological effects of sodium azide. However, he made no mention of its effect on hypertensive patients. Page and Olmsted(3), as an incidental finding in studies on vascular reactivity, mentioned the greater susceptibility of dogs with either renal or neurogenic hypertension to the acute hypotensive effects of sodium azide.

In view of the finding that sodium azide, a known metabolic inhibitor(4), produced a more pronounced lowering of blood pressure in hypertensives than in normotensives, it appeared of direct interest to use sodium azide as a tool for investigating hypertensive disease in man and experimental animals. Furthermore, the possibility was entertained that repeated administration of azide to hypertensives might so alter tissue metabolism as to result in a sustained lowering of the blood pressure. Although the drug is usually considered to be highly toxic, it should be noted that the lethal range (10 mg/kg subcut.)(5) is far in excess of the oral dosage required to produce a fall in blood pressure (.01-.02 mg/kg).

Materials and methods. A comparison was made between the effects of sodium azide in normotensive and hypertensive individuals. Included in the normotensive series are normal healthy controls (students, laboratory personnel), as well as patients suffering from diverse types of cancer. The patients with

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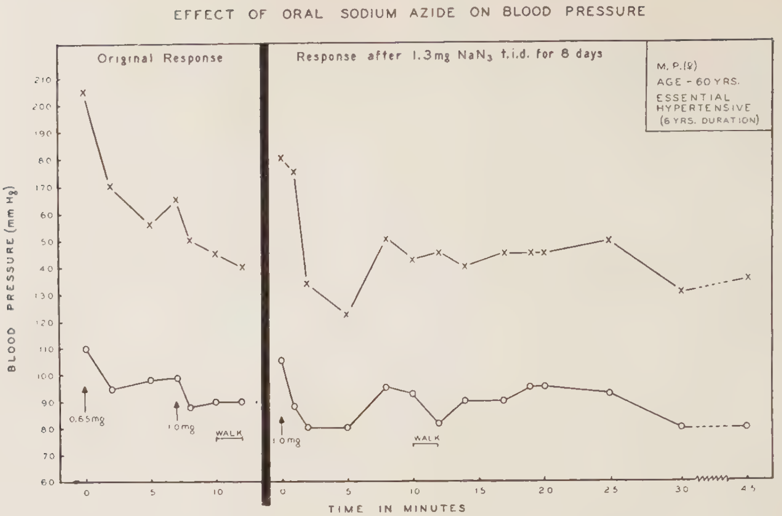

FIG. 1. Acute hypotensive action of sodium azide (oral) on blood pressure of hypertensive patient M. R. Blood pressure recorded in sitting position 205/110. Effect on B.P. with first administration of drug, as indicated in graph on left hand side of figure, remained at this level for 15 min. before returning to the original hypertensive level. Following continued treatment with azide for 8 days, the acute hypotensive effect persisted for more than 45 min., especially noticeable in diastolic pressure readings.

elevated blood pressures were documented cases of essential hypertension, recorded from 12 months to as long as 10 years. Several patients with renal parenchymatous damage were also studied. Sodium azide was administered orally, except in selected cases where a comparison was made of the response to sublingual and intravenous administration. *Blood pressure* measurements were made by the conventional inflated cuff procedure, with the patient in a sitting position. Several readings were obtained prior to the administration of the drug, after basal conditions had been established. The lowest of 3 blood pressure determinations was taken as the basal pretreatment level. Repeated blood pressure readings rarely differed by more than 15 mm Hg for the systolic values. Determinations were made at 2- to 5-minute intervals following the *acute administration* of sodium azide until the pressure had either returned to its original level, or had stabilized at a new level. Because of the rapidity of the fall in blood pressure, these necessarily represent single readings for each value recorded (Fig. 1). Sodium azide, which is tasteless in aqueous solution, was administered without informing the patient of either the nature of the drug, or the change to be expected. Doses of 0.65 mg and 1.3 mg dissolved in half or quarter of a glass of water were used in the initial experiments. Later it was found that the prescribed dosage in a teaspoon of water was equally effective. In addition to a study of the acute changes produced by sodium azide, the effects of *chronic administration* were observed in a selected group of patients. In this series, the patients were instructed to take the sodium azide orally (0.65-1.3 mg) 3 to 5 times a day.

Results. I. *Acute effects.* Oral administration of sodium azide to hypertensive patients resulted in a rapid fall in blood pressure. In some patients a hypotensive effect was observed as early as 45 to 60 seconds after the drug had been taken. Fig. 1 illustrates the character and the rapidity of the change in blood pressure following several doses of sodium azide. After testing the acute response to azide, the patient, M.R., was placed on a regime of 1.3 mg orally t.i.d. When examined 8 days later, the basal pressure was 180/105. A hypotensive response to the acute administration of azide was again obtained, as depicted in the accompanying graph (Fig. 1). After 36 days, the hypotensive response to 1.3 mg of azide was even more pronounced than originally. Several features of the response of different patients to sodium azide deserve comment. Although the actual fall in systolic pressure in mm Hg was considerably greater than the change in diastolic pressure, it should be noted that the diastolic pressure more often approached normal values than did the systolic. The fall in blood pressure was not accompanied by any significant change in pulse or respiratory rate. Flushing of the face was not observed. The majority of patients did not develop orthostatic hypotension, even after exercise. It is pertinent to note that exercise during the acute hypotensive phase did not cause any significant elevation in pressure; in fact, most patients exhibited a further lowering of diastolic pressure (5-10 mm Hg). The acute effects of sodium azide, in doses of 0.65 mg and 1.3 mg on the systolic and diastolic pressures of normotensive and hypertensive subjects are summarized in Fig. 2 and 3. The hypotensive action of azide appeared to be more closely related to the height of the initial blood pressure than to the dose level of the drug. In 13 patients whose initial systolic pressures were equal to or greater than 190 mm Hg, the acute drop in pressure averaged 43 mm Hg (range —30 to —65 mm Hg). In contrast, in 9 relatively normotensive individuals, whose systolic pressures were below 140, only a minor effect on the blood pressure occurred—a mean value of —4 mm Hg (range +12 to —22 mm Hg). A similar relationship was also observed with regard to the effect of azide on the diastolic pressure. No apparent refractoriness to the drug developed with repeated administration, comparable and even greater falls in blood pressure being elicited when the azide was administered acutely as many as ten to twelve times to the same patients at intervals of several days to several weeks. Several patients were studied repetitively for the acute effects of sodium azide for periods in excess of one year.

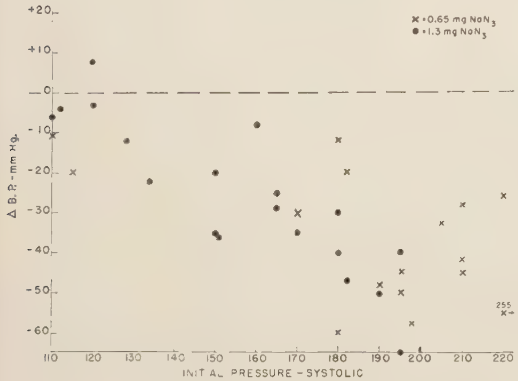

FIG. 2. Acute effect of oral sodium azide on B.P. of 35 patients plotted in relation to original systolic pressures. The change in blood pressure indicated represents maximum fall after administration of the drug. Depressant action of sodium azide was greatest in individuals with highest basal B.P.

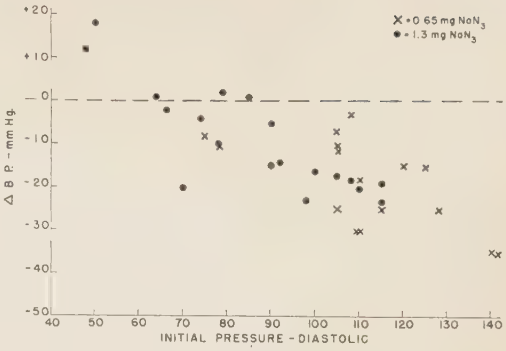

FIG. 3. Acute effect of sodium azide on B.P. of 35 patients (as in Fig. 2) plotted in relation to original diastolic pressures. Hypotensive action most pronounced in patients with highest initial values.

II. *Chronic effects.* Observations have also been made on a group of 39 patients who received sodium azide over periods varying from 5 days to more than 2 years. Of this group, 9 served as normotensive controls (diastolic pressures less than 90 mm Hg), while the remaining 30 had varying degrees of systolic and diastolic hypertension. Neither the diet nor physical activity was restricted during the study. The administration of as much as 1.3 mg of sodium azide 3 times daily for ten days to normotensive individuals had no sustained effect on the blood pressure. The effects of sodium azide on 30 hypertensives are indicated in Table I. The blood pressure values represent the reproducible reading (at least 3 to 4 times) obtained under standard basal conditions in the sitting position after the patient had been allowed to rest for 5 minutes. Blood pressure was routinely recorded from 4 to 12 hours after the last dose of azide had been taken. The response of these patients

TABLE I. Effect of Sodium Azide on Blood Pressure of Hypertensives.

Azide			Blood pressure			
				Post-treatment		
Patient	Dose (mg)	Duration (days)	Pre-treatment	Acute	Chronic	Remarks
O. D.	1.3, q.d.	5	270/140	200/110	187/112	Discontinued because of severe epistaxis
N. P.	1.3, t.i.d.	7	205/110	140/ 90	180/105	
E. F.	.7, q.d.	7	176/108	176/108	162/ 95	
G. F.	.4, t.i.d.	7	186/ 90	140/ 80	155/ 88	
M. R.	1.3, "	10	205/110	145/ 90	195/ 95	
P. S.	.65, q.d.	10	220/140	—	165/140	B.P. varied during day; renal parenchymatous damage
C. M.	1.3, by i.v. inj., q.d.	10	150/ 90	115/ 75	115/ 75	
F. G.	1.3, *idem.*	10	165/116	140/ 92	130/ 95	
G. C.	.65, t.i.d.	10	195/108	155/ 85	140/ 90	
D. E.	1.0, "	14	210/140	161/ 85	155/ 76	
L. W.	.65, "	15	210/120	182/110	178/100	
E. L.	1.3, "	17	215/135	142/102	210/130	Renal parenchymatous damage
J. S.	.65, "	18	210/130	158/195	175/105	
S. W.	1.3, "	21	250/105	205/ 95	250/105	Advanced arteriosclerosis
B. A.	.3, q.d.	21	186/104	115/ 68	120/ 80	B.P. remained normal 7 wk post-treatment
G. H.	1.3, t.i.d.	21	200/120	160/110	160/100	Previously treated with apresoline unsuccessfully
A. I.	.65, "	21	160/112	143/ 75	145/ 85	
H. S.	.65, "	21	210/110	160/ 90	160/ 90	B.P. remained normal for 14 days post-treatment
B. C.	.65, "	30	195/110	140/110	135/ 95	
G. S.	.65, "	30	180/110	140/ 90	140/ 90	B.P. remained at low level for several weeks after drug
M. K.	1.3, "	30	260/105	200/ 80	260/100	
H. K.	.5, "	30	182/100	135/ 84	145/ 95	Sympathectomy 7 yr previous
V. G.	.5, "	60	170/115	140/ 90	150/ 95	
A. S.	1.3, "	60	280/128	195/ 95	234/110	
M. W.	.65, "	90	198/110	144/ 92	160/ 90	
F. R.	.65, "	250	195/110	150/ 85	135/ 80	Off medication 2 mo—B.P. unchanged
F. S.	.65, "	275	175/110	145/ 90	130/ 90	
R. K.	1.3, "	365	200/120	125/ 88	155/100	
E. M.	1.3, "	1.5 yr	220/125	190/100	175/ 90	B.P. remained at low level for 4 mo after drug withdrawal
B. M.	.65, "	2.5 yr	180/100	120/ 70	160/105	3 yr postsympathectomy—showed increased sensitivity to azide, 0.3 mg drops B.P. to 70/40

fell into 3 categories: (1) Five cases showed only minimal changes in blood pressure after chronic administration. (2) In 10 patients the blood pressure showed a significant fall with azide, but the diastolic pressure remained above 100 mm Hg. (3) In the remaining 15 hypertensive patients, both the systolic and the diastolic pressure was maintained at near normotensive levels throughout the period of medication. Treatment with azide brought about a fall in blood pressure to normotensive levels which persisted for periods ranging from 14 days to 2 months, in 5 of the patients after the drug had been withdrawn. In these 5 cases some doubt may be entertained as to the validity of the blood pressure lowering effects of the sodium azide *per se*. It should be emphasized that all 5 cases had well-documented histories of persistent hypertension for periods of at least 2 years. Also it should be noted that at the onset of treatment, all these hypertensives exhibited a typical blood pressure response to the acute administration of sodium azide. The more common occurrence (22 out of 26) was a gradual return of the blood pressure within 1 to 3 days to hypertensive levels after withdrawal of the drug. During the period of sodium azide treatment, a majority of patients showed a definite improvement in their subjective symptoms, especially as regards headache, chest pains, and general mental outlook.

III. *Toxicity.* Three patients have been taking sodium azide daily for more than one year without evidence of damage to organs, such as the kidney, heart, liver on the basis of routine clinical studies. Kidney function was not impaired as evidenced by the quality and quantity of urine output, blood urea N and NPN. In contrast to the reported effects with hexathonium(6,7), there was no evidence of a change in bowel habits or urinary function. An occasional patient complained of a transient sensation of pounding in the head shortly after taking the drug. In 20 of the patients, continued treatment with sodium azide was followed by an increased sensitivity to the drug, necessitating a reduction in the daily dosage from 0.5 to 0.25 mg t.i.d.

IV. *Mode of administration.* Because of the unusual rapidity with which the decline in blood pressure developed following oral azide, studies were carried out with several different methods of administration. Sodium azide was administered intravenously as an infusion of 5% glucose solution containing 3.0 γ/ml of the drug. It was found that an infusion rate of 10 γ per ten minutes sufficed to lower and to maintain the blood pressure of a hypertensive patient from a level of 190/110 to 150/85 mm Hg for a period of several hours. Sublingual administration was introduced on the premise that this route might provide a more ready absorption of azide into the blood stream than oral *per se.* Tests performed on 3 hypertensives indicated that three drops containing a total of 0.1 mg by sublingual route were as effective as 0.65 mg orally.

V. *Animal experiments.* Experiments were carried out on normotensive and hypertensive rats (renal) in an attempt to clarify the mechanism of action of sodium azide. Several pertinent observations warrant discussion at this time. As in man, azide lowered the blood pressure of renal hypertensive rats from a level of 180-200 mm Hg (mean pressure by direct cannulation) to 120-130 mm Hg. As little as 0.1 mg of azide (150-180 g, body weight) was sufficient to produce a fall in blood pressure of 30 to 45 minutes duration in animals made hypertensive three months previous by either a figure-of-eight loop on the kidney or by partial ligation of the renal artery. The intravenous administration of comparable doses of azide to normotensive rats, either acutely or by slow infusion, had no effect on blood pressure. In contrast to other hypotensive drugs, azide, administered to rats which had been subjected to hemorrhage until a blood pressure of 40 mm Hg was achieved, produced no deleterious effect on either the blood pressure or the peripheral circulation as visualized in the mesentery. Sodium azide (.01-.5 mg), applied to the surface of the mesentery, had no direct dilating action on the constricted blood vessels in the terminal vascular bed of rats subjected to hemorrhage.

Discussion. Sodium azide has been found to be more effective in lowering the blood pressure from hypertensive toward normotensive levels than it is in dropping the blood pressure below control levels in normotensive individuals. The blood pressure was lowered in patients with essential hypertension, as well as in several with elevated blood pressure associated with renal parenchymal damage. Comparable hypotensive effects were observed in hypertensive rats treated both acutely and chronically with the drug. Repetitive administration on the drug over periods of 10 days or longer resulted in the appearance of an increased effectiveness in an appreciable percentage of patients tested, manifested by a greater hypotensive effect following equivalent doses and/or progressively more sustained periods of normotensive levels. In the present series it was not possible to predict on the basis of previous clinical history which of the patients would respond best to sodium azide.

In view of the small size of the present series of cases treated with sodium azide, it should be emphasized that its clinical evaluation must await considerable further investigation, especially in terms of the mode of administration and the possible use of other azide derivatives. The necessity remains to rule out all possible toxic effects of long continued administration of sodium azide before routine use in the treatment of hypertension is attempted. Because of its known effects on specific enzyme systems *in vitro,* such as cytochrome oxidase(8), catalase, peroxidase, carbonic anhydrase, and possibly transphosphorylation(9), sodium azide provides an interesting metabolic tool to investigate the pathogenesis of hypertension along biochemical lines.

Summary. 1. A comparison was made between the effects of sodium azide in both normotensive and hypertensive patients. Doses of 0.65-1.3 mg, administered orally, had a rapid hypotensive effect which persisted from 10-15 minutes. When given chronically to hypertensives (0.6-1.3 mg 3 to 4 times daily for periods up to 2 years), sodium azide produced a sustained lowering of the blood pressure toward normotensive levels. Repeated administration of the drug results in a greater hypotensive effect following equivalent doses and/or a progressively more sustained period of normotensive blood pressure levels. The observations indicate that a significant difference exists in the relative sensitivity of hypertensive and normotensive individuals to the hypotensive effects of sodium azide. 2. Intravenous injection of the drug to animals subjected to hemorrhage had no dilating effects on the constricted blood vessels in the exteriorized mesentery. Sodium azide (0.5 mg/100 g body wt) lowers the blood pressure of hypertensive rats. The observation that sodium azide in hypertensives rarely lowers the elevated blood pressure below normotensive levels suggests a general non-specific action of the drug on all vascular beds.

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1. Black, M. M., Kleiner, I. S., and Bolker, H., *Cancer Res.*, 1947, v7, 818.

2. Graham, J. D. P., *Brit. J. Pharmacol.*, 1949, v4, 1.

3. Page, I. H., and Olmsted, F., *Circulation*, 1951, v3, 801.

4. Sumner, J. B., and Somers, G. F., *Chemistry and methods of enzymes*. Academic Press, New York, 1952.

5. Handler, P., *J. Biol. Chem.*, 1945, v161, 53.

6. Page, I. H., *J.A.M.A.*, 1951, v147, 1311.

7. Hirschleifer, I., Schwarz, G., Fishman, S. I., and Frankhauser, A. M., *N. Y. State J. Med.*, 1953, v53, 1223.

8. Keilin, D., and Hartree, E. F., *Nature*, 1934, v134, 933.

9. Keilin, D., *Proc. Royal Soc.*, 1936, v121b, 165.

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Effect of 17-Vinyltestosterone on the Mammae, Uteri, Thymus, and Adrenal Glands of Spayed Female Mice. (20771)

D. S. FLUX. (Introduced by R. Gaunt.)

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17-vinyltestosterone appears to be an interesting steroid in that it shows evidence of androgenic activity (Kochakian(1), Leathem (2)) and also indications of corticoid activity (Lewis, de Majo & Rosemberg)(3) but did not support lactation in adrenalectomized rats in doses up to 2 mg daily (Flux)(9). It is closely related structurally to ethinyl testosterone (pregneninolene, anhydrohydroxy progesterone) which has progesterone-like, oestrogenic and androgenic activities (Emmens & Parkes(4), Chamorro(5-7)). Since androgens and at least one 11-deoxycorticosteroid, 11-deoxycorticosterone acetate (see review by Folley(8)), have been found to promote growth of the mammae in experimental animals, it was decided to investigate the effect of 17-vinyltestosterone on the mammary glands of spayed mice, and in addition to collect data on its effects on body weight, uterus weight and on the weight of the thymus and adrenal glands.

Methods. Female mice of the CHI strain were ovariectomized at 14 days of age and weaned at 21 days. Mice in one group of 6 animals were each subcutaneously implanted with one 100 mg tablet of 17-vinyltestosterone, those in another group were each given four 50 mg tablets and 6 otherwise untreated ovariectomized mice and 6 intact mice were used as controls. The animals were killed at 42 days of age and the uteri and the thymus and adrenal glands were weighed. The uteri were split and blotted to remove any fluid in the lumina. Whole mounts of all the mammary glands were prepared and the total area covered by each set of glands was determined by placing the slides in a projector and measuring the area covered by the image of the separate glands with a planimeter and summing for each mouse. To obtain an objective measure of the degree of branching of the duct systems the images of the first 2 pairs of thoracic glands were projected and the number of junctions between ducts counted. In the case of the larger and more complex duct systems the images were projected onto squared paper (one inch squares for larger and half inch for smaller glands) and the number of duct junctions in a quarter (with a minimum of eight) of the squares covered by the image were counted. These, together with the number of squares covered, made it possible to estimate the number of duct junctions per gland.

Results. The results are shown in Table I. The mice implanted with one 100 mg tablet vinyltestosterone absorbed an average of 0.88 mg per day and those given four 50 mg tablets absorbed almost twice as much, 1.61 mg daily. Although the mean body weights of the mice given vinyltestosterone were higher than those of the mice in the control groups the differences were not significant. At both dose levels vinyltestosterone caused an increase in the mean total area of the mammae over that of the ovariectomized control mice but neither approached that of the intact control mice. The number of duct junctions was increased in the treated mice and in those given the higher dose the mean number of duct junctions per unit area was considerably greater than in the other groups, including intact controls. No alveoli were seen on any of the duct systems. Vinyltestosterone caused considerable increases in the weight of the uteri, although the means did not reach that of the intact controls. It also caused a depression in thymus weight and in the case of the higher dose, in adrenal gland weight. In all cases the effect of the higher dose exceeded that of the lower.

Discussion. 17-vinyltestosterone was similar to the closely related compound ethinyl testosterone (pregneninolene) tested by Chamorro(5-7) in that it promoted growth of the

TABLE I. The Effect of 17-Vinyltestosterone, Given from 21 to 42 Days of Age on Mammary Glands of Female Mice Spayed at 14 Days. (6 mice in each group.)

Treatment	Mean body wt (final), g	Mammary gland: Total area, mm²	Mammary gland: Total area, Log₁₀ (mm²)	Mammary gland: No. of duct junctions*, Total	Mammary gland: No. of duct junctions*, Log₁₀ (total)	Mammary gland: No. of duct junctions*, Per 10 mm²	Mean uterus wt, mg	Mean uterus wt, Log₁₀ (mg)	Mean thymus wt, mg	Mean adrenal wt, mg
Spayed controls	18.2	58	1.763	71	1.850	26.3	9.2	.954	62	7.0
Spayed + vinyltestosterone										
1 × 100 mg tablet (.88 ± .09 mg daily)	20.6	85	1.924	184	2.234	42.8	37.0	1.554	48	5.9
4 × 50 " " (1.61 ± .09 " ")	20.0	194	2.282	695	2.826	73.6	59.0	1.768	36	5.3
Intact controls	17.6	599	2.764	1137	3.040	45.5	98.0	1.968	53	8.2
Min difference between means										
Significant at 5% level	No diff.		0.105		.522	10.4		.152	10.2	1.6
" " 1% "	Sig.		0.150		.743	14.7		.216	14.6	2.2

* Counted: the 1st and 2nd pairs of thoracic mammary glands.

mammary duct systems of spayed female mice. Differences in numbers of duct junctions per unit area must be interpreted cautiously because, in the mammae of intact or oestrogen-treated spayed mice the density of duct junctions is not related in a simple manner to the area of the duct system (Flux)(9). However the number of duct junctions per unit area in the mammae of the mice treated with vinyltestosterone was much greater than in those of comparable sizes in either intact or oestrone treated spayed mice in other experiments (Flux)(9) and it appeared certain that vinyltestosterone stimulated the growth of side branches in the duct systems. As far as effects on the weight of the uteri, and thymus and adrenal glands of spayed mice were concerned the results of this experiment were in accordance with those of Kochakian(1) and Lewis *et al.*(3).

Summary. 17-vinyltestosterone promoted growth in the extent and in the degree of branching of the mammae of spayed female mice but did not cause growth of alveoli on the duct systems. It also caused increases in the weight of the uteri of spayed mice and depressed the weights of the thymus and adrenal glands.

I am indebted to Dr. S. J. Folley for his interest in this experiment which was carried out in his laboratory and to Ciba Ltd., Horsham, England, through the courtesy of Dr. C. W. S. Taylor, for vinyltestosterone given to Dr. Folley. During the course of the work I received financial support from Dairy Research Institute, New Zealand, and from Department of Scientific and Industrial Research (N.Z.) as a National Research Fellow.

1. Kochakian, C. D., *Recent Progress in Hormone Research,* ed. G. Pincus, 1947, v1, 177.

2. Leathem, J. H., *Trans. New York Acad. Sci.,* Series II, 1950, v12, 234.

3. Lewis, R. A., de Majo, S., and Rosemberg, E., *Endocrinology,* 1949, v45, 564.

4. Emmens, C., and Parkes, A. S., *Nature,* Lond., 1939, v143, 1064.

5. Chamorro, A., *C. R. Soc. Biol.,* Paris, 1943, v137, 87.

6. ———, *ibid.,* 1944, v138, 71.

7. ———, *ibid.,* 1945, v139, 958.

8. Folley, S. J., in *Marshall's Physiology of Reproduction,* 1952, v2, ed. A. S. Parkes, Longmans.

9. Flux, D. S., Thesis, University of Reading, 1953.

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Effect of Aureomycin in Preventing Liver Abscess in Cattle.* (20772)

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Liver abscesses result in the condemnation of about 5% of the livers from cattle slaughtered in Federally inspected meat packing plants in the United States. This loss amounts to more than $2,000,000 annually. According to the National Livestock Loss Prevention Board(12), the regional incidence of abscesses, telangiectasis and "sawdust"† is highest in the western states. Jensen, *et al.*(8) found that liver abscesses develop in significant numbers, both in the feed lot and on the range. Abscesses appear to develop 80 to 120 days after the cattle are placed in the feed lot. These investigators also observed that telangiectasis develops early and abscesses form later in the fattening period. The studies of Smith(14) indicate that there is a definite relationship between ulcers of the rumen and abscesses of the liver in beef cattle. A recent study by Jensen(9) shows a direct relationship between liver abscesses and stomach injuries. Newsom(13) and Yamamoto (15) in separate investigations found that the predominating organism in abscessed livers was *Actinomyces necrophorus.* Merchant(11) classifies the same organism as *Spherophorus necrophorus*. The organism appears to be a normal inhabitant of animal tissues. Animals are not immunized against liver abscesses when cultured filtrates of *Spherophorus necrophorus* are given intraperitoneally(9). Since the organism is frequently a secondary invader in other diseases in which primary necrosis or destruction of tissue exists(11), this may account for its failure to be cultured under *in vivo* conditions in cattle. However, acute hepatic necrosis has been produced experimentally in rats kept on a diet low in casein (2,3) and also on rations containing yeast as the sole source of protein(7). When aureomycin was added to a necrogenic yeast diet(4) it was found to have a significantly beneficial effect by preventing experimental hepatic necrosis in rats. The assumption is made that aureomycin might act through the suppression of the intestinal flora. Abell and Bweridge(1) demonstrated that sulfaguanidine also delayed the development of liver necrosis in rats. Dietary imbalances, such as excessive fat(5,10), carbohydrate(6), or proteins (3) have not been consistent in the development of necrosis in the liver of rats.

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† A term used to designate a condition of liver characterized by many areas of focal necrosis spread throughout the liver or localized giving the appearance of sawdust on the liver.

The primary objective of the experiment in which the following results are reported was to study the effect of aureomycin in controlling calf scours. The observations of the condition of the livers were made at the time of slaughter following feeding experiments.

Methods. The Milking Shorthorn calves used in these experiments were born during the 1950-51 or the 1951-52 seasons. The newborn calves were given special aureomycin tablets or aureomycin capsules‡ orally each day for 12 weeks. The tablets and capsules were composed primarily of sugar base and contained 15 mg aureomycin. The calves were paired as evenly as possible with respect to birth weight, age, and age of dam. One calf of each pair was given an aureomycin tablet immediately or shortly after receiving colostrum. Some of the paired calves were fed milk from nipple pails while others were put on nurse cows. The antibiotic tablets or capsules were given to the test calves in both groups (bucket-fed and calves on nurse cows) at the rate of 15 mg per head daily. The animals used in the 1951-52 fattening test were given one aureomycin tablet or capsule daily during the first 12 weeks after birth while the animals in the 1952-53 feeding test were given two aureomycin tablets every other day.

Results. No case of scours was observed in the test groups which received aureomycin. The calves in the control groups which received no aureomycin scoured intermittently. Severe cases of scours were observed during the first 6 weeks following birth. Approximately 20 percent larger gains were made by the calves which received a daily intake of 15 mg of aureomycin. All of the calves were weaned at approximately 196 days of age. The animals fattened during 1951 and 1952 were put in a dry-lot and shortly after weaning they were fed a ration consisting of 60% corn, 20% oats, 13% dried beet pulp and 7% soybean oil meal. Alfalfa hay was also fed. The animals used in the 1952-53 fattening experiment were turned out to pasture during the summer of 1952 after weaning. Then they were fed a ration consisting of the same grain mixture as above with corn silage and alfalfa hay for 128 days. After this period the silage was eliminated from the ration.

The results of the feeding trials are presented in Table I.

The animals were to be slaughtered when they reached about 1000 pounds but this plan could not be adhered to strictly. Thus, it will be noted that certain animals were fed considerably longer than others.

The liver from each of the steers was examined at the time of slaughter. In several previous feeding experiments with dual-purpose steers where no antibiotics were fed, approximately 75% of the livers were found to be abscessed and those which were not abscessed showed scar tissues. When healthy, normal livers without scar tissues were observed among the group of animals slaughtered in 1952, the histories of these animals were examined. It was found that the animals which received aureomycin during the first

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‡ The aureomycin-hydrochloride for the capsules and the aureomycin tablets were supplied through the courtesy of Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE I. Feed Lot and Slaughter Data.

	Pair	Steer No.	Birth wt, lb	Initial wt, lb	Days on feed	Avg daily gain, lb	Slaughter wt, lb*	Condition of liver
				1951-1952 feeding period				
Control	1	314	74	440	314	2.02	1042	Abscess
	2	334	93	610	202	1.63	930	”
	3	337	83	350	314	2.07	999	Sawdust†
	4	313	78	460	314	1.93	1024	Abscess
	5	339	72	335	341	1.83	920	”
		Avg	80	439	297	1.90	983	
Treated	1	325	87	550	202	2.03	905	Normal
	2	308	84	450	282	2.09	940	”
	3	338	82	505	202	2.10	900	”
	4	309	77	415	289	2.02	1000	”
	5	327	63	320	341	1.91	950	Small scar‡
		Avg	79	448	263	2.03	939	
				1952-1953 feeding period				
Control	1	56	72	675	284	1.13	981	Abscess
	2	65	72	660	197	1.70	920	”
	3	61	69	620	197	2.11	985	Normal
	4	67	83	565	284	1.81	1020	Abscess
	5	73	60	460	284	2.08	973	Scar‡
		Avg	71	596	249	1.77	976	
Treated	1	58	74	655	197	2.08	970	Normal
	2	64	73	580	284	1.83	1033	”
	3	62	86	550	197	2.36	940	”
	4	70	74	525	284	1.88	997	”
	5	74	65	510	197	2.28	890	”
		Avg	74	564	232	2.09	966	

* 24-hr shrinkage.

† A term used to designate liver that contains many areas of focal necrosis spread throughout the liver giving the appearance of sawdust on the liver.

‡ Passed.

twelve weeks after birth had the normal livers, as shown in Table I. Similar observations were made in the second group of steers slaughtered in 1953.

Summary. Under the conditions of these experiments, it appears that aureomycin prevented scouring during the early preweaning period and thereby did not predispose the treated animals to necrophorus infection.

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1. Abell, M. R., and Bweridge, J. M. R., *Can. J. Res.*, 1949, v27, 316.

2. György, P., and Goldblatt, H., *J. Exp. Med.*, 1942, v75, 355.

3. ———, *ibid.*, 1949, v89, 245.

4. György, P., Stokes, Joseph Jr., Smith, W. H., and Goldblatt, H., *Am. J. Med. Sci.*, 1950, v220, 671.

5. György, P., *Med. Clin. N. American*, 1949, v33, 1657.

6. Himsworth, H. P., and Glynn, L. E., *Clin. Sci.*, 1944, v5, 93.

7. Himsworth, H. P., and Lindan, O., *Nature*, 1949, v163, 30.

8. Jensen, Rue, Frey, P. R., Cross, Floyd, and Connell, W. E., *J. Am. Vet. Med. Assn.*, 1947, v110, 256.

9. Jensen, Rue, Ph.D. Thesis. Univ. of Minnesota, June, 1953.

10. McLean, J. R., and Bweridge, J. M. R., *J. Nutrition*, 1952, v47, 41.

11. Merchant, I. A., *Veterinary Bacteriology and Virology, 4th ed.*, Iowa State College Press, 1950, p474.

12. National Livestock Loss Prevention Board, Meat Wasted in Marketing Livestock, 1944 Report.

13. Newsom, I. E., *J. Infect. Dis.*, 1938, v63, 232.

14. Smith, H. A., *Am. J. Vet. Res.*, 1944, v5, 234.

15. Yamamoto, S., *J. Jap. Soc. Vet. Sci.*, 1938, v17, 40.

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Ability of *Endamoeba histolytica* to Phagocytose Red Blood Cells.* (20773)

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The ability of *Endamoeba histolytica* to ingest red blood cells is a well-recognized characteristic. The following experiments were designed to study this property quantitatively and to determine what the effect of certain drugs and of antiserum might be on this phagocytic tendency.

Materials and methods: It was found that reproducible phagocytic indices could be obtained in the following manner: Three-tenths ml of a one in 10 dilution of defibrinated rabbit blood was added to 48-hour cultures of the 200† strain of *Endamoeba histolytica* in the Shaffer-Frye medium(1). Only cultures showing 4+ amoebae were used. The tubes were gently agitated to distribute the RBC and the petrolatum seal restored. These amoeba-RBC mixtures were then incubated at 37°C for 15 minutes. At the end of this period, most of the culture fluid was removed and discarded. Ten ml of distilled water was then gently added. This hemolysed all unphagocytosed RBC, but did not disrupt the amoebae and left the phagocytosed RBC intact. Samples were removed from the bottom of the tube and the number of RBC in 25 amoebae counted and averaged. The resulting phagocytic indices were constant to within ±1 RBC over a series of similar tubes run in a given experiment. It was found that after 30 minutes in the distilled water the RBC began to appear distorted, thus, it was important to set up the tubes individually allowing an interval between each succeeding culture to provide time for counting. The rabbit blood was collected by cardiac puncture and defibrinated by shaking with glass beads. Blood kept more than 5 or 6 days was found undesirable. Hank's balanced salt solution (Microbiological Associates, Bethesda, Md.) was used as a diluent for all reagents. All materials were brought to 37°C before addition to the cultures.

A series of experiments was next designed to study the influence of certain compounds on the ability of the 200 strain of *Endamoeba histolytica* to phagocytose rabbit RBC. Aureomycin HCl, terramycin HCl, ilotycin, fumagillin and emetine HCl were used. The first 4 compounds were the pure crystalline substances, while the fumagillin was in the form of 10 mg capsules (Abbott). The crystalline compounds were weighed on an analytical balance and dissolved to appropriate concentration in the balanced salt solution, while the fumagillin was made up to the desired volume and thoroughly emulsified from the capsule. Twelve 48-hour cultures of *Endamoeba histolytica* were used in each experiment. A series of 5 drug solutions was made to contain appropriately decreasing amounts, so that when 0.2 ml was added to a culture, the desired final concentration was reached. Each of the 5 solutions was added through the petrolatum seal to a pair of amoeba cultures and 0.2 ml of Hank's solution was added to an additional pair of cultures as controls. All were gently mixed and re-sealed. One of each pair of cultures was incubated at 37°C for 2 hours and the other member was incubated for 20 hours. At the end of the incubation period, rabbit RBC were added and the phagocytic index determined. The results of the experiments were recorded as the phagocytic index for each drug concentration and the control, and graphs made, with the drug concentrations on the ordinates and the phagocytic indices on the abscissas. In figuring the phagocytic index, only those amoebae containing RBC were included in the experiments here described. The numbers of trophozoites which did not phagocyte RBC varied greatly between similar cultures and appeared to be unrelated to drug con-

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* Aided by a grant from the National Microbiological Institute, National Institute of Health, Public Health Service.

† The 200 strain of *Endamoeba histolytica* was obtained from Mr. M. C. McCowen of The Lilly Research Laboratories. He originally obtained the culture from Dr. C. W. Rees of the National Institutes of Health.

centrations or other measurable factors. Further investigation of this is necessary.

Another set of experiments was done to test the effect of antiserum on the ability of the amoebae to phagocytose RBC. Anti-amoebic serum was produced in 2 rabbits by giving a series of 8 bi-weekly intravenous injections of concentrated suspensions of *E. histolytica,* strain 200. Each inoculation consisted of 2 to 3 million *E. histolytica* trophozoites prepared from 48-hour cultures in S-F medium by washing once with saline (0.85% NaCl) and concentrating in a volume of one to 2 ml. Blood was collected from the rabbits by cardiac puncture before the series of inoculations was started and 13 days after the final inoculation. The serum was stored in the deepfreeze. The technic was modified when testing the effect of serum on the phagocytic activity of the *E. histolytica* cultures in order to conserve serum and was as follows: Forty-eight-hour cultures of the amoebae were used as before. Just prior to addition of the serum, most of the culture fluid was removed leaving a previously determined volume so that a desired final concentration of serum could be obtained. Thus, if the effect of serum 1:8 was to be determined, 1.75 ml of culture was left and 0.25 ml of serum added. Other desired dilutions could be reached in a similar manner, keeping the final volume of fluid constant in the tubes. After addition of the serum, the cultures were sealed with vaseline and incubated at 37°C for 2 hours. At the end of this incubation period, rabbit RBC were added and phagocytic indices calculated as previously described.

Results. The results of the experiments on drugs are illustrated graphically in Fig. 1-3. Each of the graphs is a composite of 4 or 5 individual experiments with one of the compounds studied. The starred circles represent the phagocytic indices obtained after 2 hours exposure to the drug and the solid black circles represent the phagocytic indices obtained after 20 hours exposure to the drug. The broken line connects the midpoints of the 2-hour indices and the dotted line connects the midpoints of the 20-hour indices.

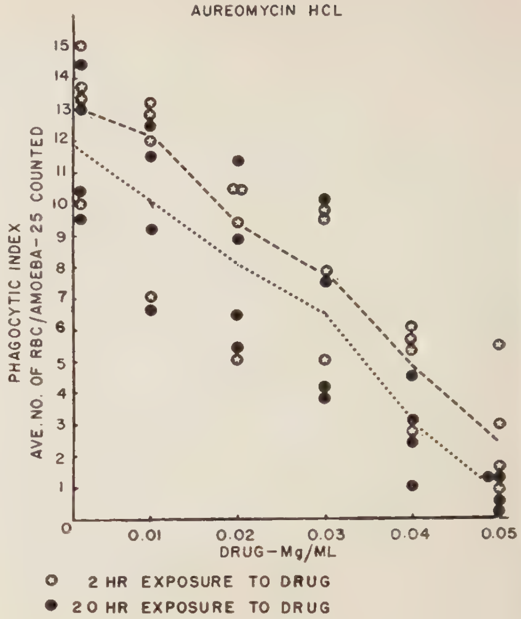

FIG. 1.

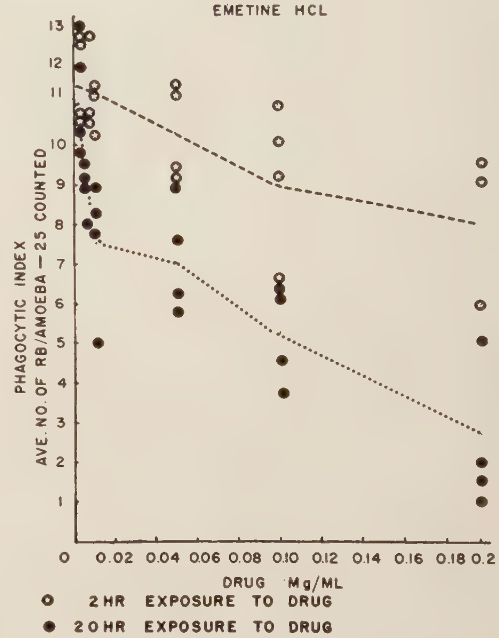

FIG. 2.

Fig. 1 shows the results obtained with aureomycin HCl. It will be seen that, as the concentration of drug was increased, the phagocytic index showed a steady decline. Essentially similar decreases occurred whether there was 2- or 20-hour exposure to the drug.

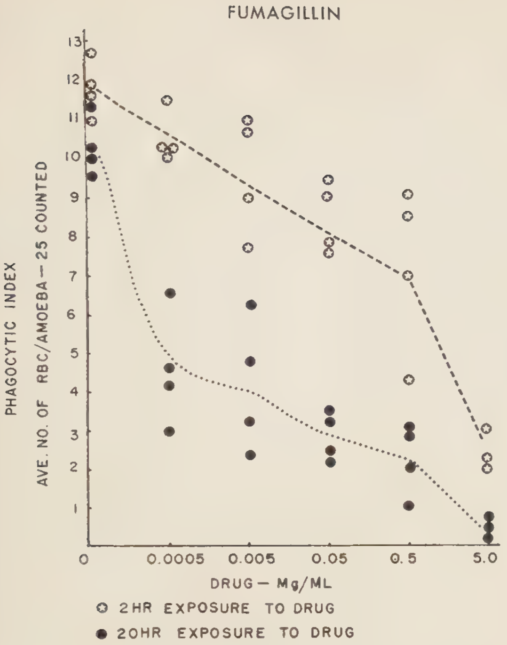

FIG. 3.

Fig. 2 shows the results obtained with emetine HCl. With this drug there was a difference between the 2- and 20-hour phagocytic indices. It was found in preliminary experiments that the range of concentrations showing little or no activity and that showing marked activity, especially at 20 hours, was rather narrow. Thus, it was desirable to use closely graded concentrations in the lower range. An examination of Fig. 2 reveals that a relatively greater reduction of phagocytic index occurred after 20 hours than after only 2 hours exposure to the various concentrations of emetine HCl.

Fig. 3 shows the results obtained with fumagillin. It will be seen that there is an even greater difference between the 2- and 20-hour results with fumagillin than observed in the case of emetine HCl. As was also true with aureomycin HCl and emetine HCl, increases in the concentration of fumagillin resulted in decreased phagocytic indices. It will be noted, however, that the range of drug concentrations exhibiting minimal and maximal activity was much greater with fumagillin than with either aureomycin HCl or emetine HCl.

Results obtained with terramycin HCl were of some interest since the general pattern fell between that of aureomycin HCl and emetine HCl. There were differences between the results on 2- and 20-hour exposure, but not as marked as those seen with emetine HCl as shown in Fig. 2. The range of this activity with terramycin HCl fell between 0.05 and 0.3 mg/ml, a relatively narrow range.

Ilotycin was found to have a pattern similar to that of aureomycin HCl, with little difference in the phagocytic indices after 2- and 20-hour exposure of the amoebae to the various concentrations. The range of this activity with ilotycin was from 0.25 to 1.25 mg/ml.

The results of the studies made using the normal and anti-*E. histolytica* (strain 200) rabbit serum with 4 different strains of *E. histolytica* are shown in Table I. These results reveal that incubation of the 4 strains of *E. histolytica* for 2 hours in the presence of the rabbit antiserum diluted 1:8 resulted in a definite reduction in the phagocytic index. The normal serum of rabbit No. 1 exerted little, if any, effect, while the normal serum of rabbit No. 2 reduced the phagocytic index moderately. Thus, the inoculation of these rabbits with suspensions of strain 200 *E. histolytica* resulted in increased ability of their serum to inhibit phagocytosis of RBC by not only strain 200, but also strains 103, Cook and K-14, *E. histolytica*. In further experiments it was found that exposure of the amoebae to the antisera diluted 1:16 had little effect on the phagocytic index. Results in a limited number of experiments with normal and "immune" serum, 1:4, were inconsistent.

TABLE I. Phagocytic Indices Obtained Following Exposure of 48-Hr Cultures of Four Strains of *E. histolytica* to Anti-Amoebic Rabbit Serum for 2 Hours at 37°C.

Rabbit serum (anti-200) (1 in 8 dilution)	Phagocytic index* *E. histolytica* strain			
	200	103	Cook	K-14
#1 Normal	12	10	11	11
"Immune"	3	3	2	4
#2 Normal	9	9	9	9
"Immune"	3	4	3	3
Control (no serum)	13	10	14	10

* Avg No. of RBC/amoebae in a total of 25 amoebae.

Since the antisera were produced by inoculation of *E. histolytica* grown in the S-F medium which depends on the presence of bacterial cells of a gram negative anaerobic streptobacillus, it was desirable to adsorb the sera with concentrated suspensions of these bacteria to see if this affected the ability of the serum to reduce the phagocytic index. The adsorbed "immune" and normal sera exhibited the same activity as similar unadsorbed sera. So far, no tests have been made using anti-streptobacillus serum.

Discussion. The significance of the observations recorded in Fig. 1-3 is not yet known. It seems likely that the results reflect a variation in the behavior of the compounds involved. This variation may be in terms of fundamental differences in the point of attack on the amoebae, selective effects on the most actively phagocytic cells by aureomycin HCl, but not emetine HCl and fumagillin, or any one of a number of other possibilities. Further experimental and statistical examinations are necessary before definite conclusions can be reached.

Tabulation of the distribution of RBC per amoeba in the individual experiments has revealed some interesting data. The number of RBC per trophozoite varies from none to as high as 30. Plotting of these figures indicates a normal distribution with a low peak at the level of the median which has in all calculated cases been within one of the mean or phagocytic index. Consequently, the standard deviation is likely to be high. The reasons for the variation in numbers of phagocytosed RBC in individual amoebae are not known. It may be that this is a reflection of the metabolic or developmental state of the individual organisms. The irregularity of phagocytic activity exhibited by older cultures may present evidence favoring this viewpoint.

Insufficient experiments have been performed with anti-amoebic rabbit serum to determine the nature of this activity. It would seem likely that an antibody to some surface component of the cell is present in the serum as a result of the inoculation of the rabbits. The results shown in Table I show that all 4 strains of *E. histolytica* tested were affected by the serum. The technic is not yet standardized to the point where it can be determined whether or not there are antigenic differences between the strains. Further experiments, designed to produce higher titered sera and to further standardize the procedure, are in progress.

Cole and Kent(2) have recently published the results of amoeba-immobilization tests in which they tested the effect of anti-amoebic rabbit serum and the serum of human patients with amoebiasis for ability to cause *E. histolytica* to round up. It would seem that immobilization might be reflected directly in loss of ability to ingest particles, thus the two methods appear likely to be testing similar phenomena.

It is felt that the methods described above may offer an objective approach in studying certain fundamental characteristics of *E. histolytica* and in understanding the mode of action of anti-amoebic drugs and of antiserum. The technic itself needs further study and standardization as indicated before. It may be that the phagocytic index is not the most desirable unit to use, but this can only be determined by future experiments.

Summary. 1. A method for determining phagocytic indices for *E. histolytica* with rabbit red blood cells has been described. 2. The effect of several drugs on the ability of *E. histolytica* to phagocytose rabbit RBC has been tested and recorded. 3. All drugs tested were found capable of reducing the ability of the amoebae to phagocytose rabbit RBC. A range for this activity was established under the conditions used. 4. Certain differences in the effect of the different compounds were observed. 5. The effect of anti-amoebic serum on the phagocytic indices of 4 strains of *E. histolytica* was tested. It was found that definite reductions could be obtained.

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1. Shaffer, J. G., *Am. J. Hyg.*, 1952, v56, 119.

2. Cole, B. A., and Kent, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 811.

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Antibacterial Action of Tetracycline: Comparisons with Oxytetracycline and Chlortetracycline.*† (20774)

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The importance of minor changes in chemical structure in affecting the antimicrobial action as well as the pharmacologic and toxic properties of substances is well recognized in the field of chemotherapy. The preparation of tetracycline(1,2) by the catalytic hydrogenation of chlortetracycline (Aureomycin) offered an opportunity to compare the properties of 3 closely related antibiotics, each varying from the other by a single atom. Tetracycline has a structure common to both chlortetracycline and to oxytetracycline (Terramycin), lacking both the chlorine atom which is present on the aromatic ring of chlortetracycline(3) and absent from oxytetracycline (4) and an OH group which distinguishes oxytetracycline but which is also absent from chlortetracycline. Comparisons of the antibacterial action of these three tetracycline analogues are presented in this paper.

Materials and methods. Tetracycline (supplied in the latter part of this study under the trademark Achromycin) and chlortetracycline (Aureomycin) were furnished by Lederle Laboratories through the courtesy of Dr. Stanton M. Hardy. Oxytetracycline (Terramycin) was provided by Chas. Pfizer & Co. through the kindness of Dr. Gladys L. Hobby. These three antibiotics were each made available as crystals of the hydrochloride in sterile, rubber-capped vials containing 100 or 500 mg equivalent of the base. The strains of bacteria studied were from various infected materials obtained from patients in the hospital; most of them were isolated and identified in the bacteriological laboratory of the Mallory Institute of Pathology by Miss Marion E. Lamb and Miss A. Kathleen Daly. The strains of *Neisseria gonorrhoeae* were isolated by Mrs. Helen Trousdale of the Genitourinary Clinic, and most of the strains of *Neisseria meningitidis* were furnished by Dr. Sara E. Branham of the National Microbiological Institute. Tests for sensitivity were carried out by streaking a loopful of freshly grown broth culture onto the surface of each of a series of plates of heart infusion agar (Difco pH ± 7.4) containing 2-fold dilutions of each of the antibiotics. Visible growth was recorded after 24 hours and again after 48 hours of incubation at 37°C, except in the case of Neisseria which were read only after 48 hours. Horse blood was incorporated in the agar for the pneumococci, streptococci, staphylococci and Neisseria. The sensitivity of a strain was considered to be the minimum concentration of antibiotic which produced marked inhibition of growth at 24 hours. In making comparisons, however, the complete inhibition (no detectable growth) after 48 hours was also used. Growth curves were carried out with cultures of *Bacillus cereus* No. 5 (obtained from Dr. A. C. Dornbush of the Lederle Laboratories) and *Streptococcus 98* in brain heart infusion broth (Difco pH ± 7.4), with and without various concentrations of the three antibiotics, and determining the number of viable organisms at appropriate intervals in pour plates of the cultures in antibiotic-free agar. The seed cultures had been grown overnight in broth and horse blood was incorporated in all cultures of *Streptococcus 98*. Assays for each of the 3 tetracyclines in serum and urine, and also for standard solutions of these antibiotics were carried out with *B. cereus* No. 5 by a serial two-fold dilution method in broth.

Results. Effect of storage in the cold. Citrated plasma from venous blood, in amounts

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* Summary of some of these findings presented at the Antibiotic Symposium, Washington, D.C., Oct. 28, 1953.

† Aided by a grant from the U. S. Public Health Service.

‡ The authors are indebted to Clare Wilcox and Ann Najarian for technical assistance.

TABLE I. Sensitivity of 257 Recently Isolated Strains of Common Pathogenic Bacteria to 3 Tetracyclines.

Organism	No. of strains tested	Antibiotic*	Minimum inhibiting concentration (M.I.C.), µg/ml															M.I.C. for ⅔ of strains
			>200	200	100	50	25	12.5	6.3	3.1	1.6	.8	.4	.2	.1	.04	.02	
D. pneumoniae	22	T										1	1	14	6			.2
		O											1	9	10	1		.2
		C										1	2	10	4	4	1	.2
Strep. viridans	15	T										1	8	3	2	1		.4
		O										1	8	2	2	1	1	.4
		C										1	0	10	3	0	1	.2
Strep. hemolyticus	24	T				2	0	1	5	7	5	4						6.3
		O				1	1	0	9	5	5	3						6.3
		C				2	0	9	4	2	6	1						12.5
Enterococcus	14	T	8	0	0	0	0	0	5	1								>200
		O	6	1	1	0	0	1	4	1								>200
		C	2	1	3	2	0	0	1	0	5							100
Staph. aureus	17	T	6	0	0	0	0	0	0	1	4	5	1					3.1
		O	6	0	0	0	0	0	0	0	7	3	1					3.1
		C	3	2	0	1	0	0	0	0	1	10						1.6
N. gonorrhoeae†	38	T								2	4	7	15	10				.8
		O									3	6	23	6				.4
		C								8	10	14	3	3				1.6
N. meningitidis†	24	T								6	12	6						1.6
		O								5	11	8						1.6
		C					1	3	5	15								6.3
E. coli	28	T							12	8	8							6.3
		O							10	8	7	3						6.3
		C					4	7	17									12.5
A. aerogenes	21	T	1	0	2	3	4	2	3	3	2	1						25
		O	1	0	1	3	7	1	0	3	3	2						25
		C	1	0	3	5	1	2	2	4	1	2						50
Shigella sonnei	14	T						7	5	2								12.5
		O						7	7									12.5
		C					7	0	7									25
B. proteus	6	T	2	2	1	1												200
		O	6															>200
		C	5	1														>200
P. aeruginosa	34	T	4	9	10	5	2	1	0	0	0	3						200
		O	2	3	18	3	1	1	1	1	1	3						100
		C	14	6	5	1	1	4	0	1	1	1						>200

* T = tetracycline; O = oxytetracycline; C = chlortetracycline.

† Tests of these organisms were read after incubation for 48 hr; all others were read at 24 hr.

sufficient for several replicate assays, was obtained from 6 patients after oral or intravenous administration of tetracycline. Similar samples of plasma were also obtained at several intervals following ingestion of 0.5 g of one of the 3 tetracyclines given to 6 subjects, each antibiotic being given to 2 of them. Samples of urine were also obtained from the same subjects. Assays for the antibiotic content of the plasma and urine were carried out with these samples promptly and the assays were repeated after they had been stored in the frozen state at –25°C for varying periods up to 3 weeks. No deterioration in antibiotic activity of these specimens could be determined by this test. Standard solutions of 2 μg/ml of each of the 3 antibiotics were also prepared in broth; these were tested promptly and again at intervals up to 3 weeks of storage at 5° to 10°C (in a commercial refrigerator) and at –25°C (in a commercial "deep-freeze"). The solutions all retained their activity essentially unimpaired during the 3 weeks of storage at –25°C. At 5° to 10°C the chlortetracycline solution lost about 50% of its activity (difference of one dilution tube) between the second and third day, and 75% of its activity was lost (a difference of 2 dilution tubes) between the 4th and 7th day; the oxytetracycline lost 50% of its activity between the 4th and 7th day but the tetracycline solution showed no loss of activity as demonstrated by this test during 3 weeks of storage.

Sensitivity of recently isolated strains. The results of tests for sensitivity of 257 strains of common pathogens carried out simultaneously with each of the 3 tetracycline analogues are shown in Table I. On the whole, the strains of any given species or genus varied in their sensitivity to the 3 tetracyclines over a similar range of concentrations, and the minimum concentration required to inhibit two-thirds of the strains of each species or genus that was tested likewise did not differ widely except in the case of Neisseria for which the minimum inhibiting concentration of chlortetracycline was greater than for the other 2

TABLE II. Comparison of Sensitivities of Individual Strains to 3 Tetracyclines.

Organisms	No. of strains tested	Hr of incubation*	$\frac{\text{M.I.C. oxytetracycline}}{\text{M.I.C. tetracycline}}$							$\frac{\text{M.I.C. chlortetracycline}}{\text{M.I.C. tetracycline}}$						
			8	4	2	1	½	¼	⅛	8	4	2	1	½	¼	⅛
D. pneumoniae	22	24				16	5	1				3	11	6	1	1
		48				11	10	1				3	14	4	1	
Strep. viridans	15	24			1	10	4						6	7	1	1
		48			1	9	5				4	4	6	1		
Strep. hemolyticus	24	24		3	4	11	5	1		3	4	8	5	3	1	
		48		4	5	6	5	3	1	5	3	6	9	1		
Enterococcus	14	24			1	11	0	1	1			1	3	5	3	2
		48			1	11	1	1			6	0	6	2		
Staph. aureus	17	24			3	12	2					1	8	7	0	1
		48			5	12				9	2	0	5	1		
N. gonorrhaeae	38	48			8	19	6	5		2	16	13	5	1	1	
N. meningitidis	24	48		1	3	12	8			4	10	5	5			
E. coli	28	24	2	2	6	7	3	8		7	6	9	6			
		48		6	4	6	9	3		14	9	5				
A. aerogenes	21	24		1	1	12	7				1	5	11	3	1	
		48			4	10	6	1			9	7	5			
Shigella sonnei	14	24			2	12						9	5			
		48			7	7					7	7				
B. proteus	6	24	4	2						1	1	1	3			
		48	4	2						2	1	3				
P. aeruginosa	34	24		1	3	13	11	4	2		9	9	12	2	2	
		48		1	0	9	22	2			4	6	22	2		
All strains	195	24	6	9	21	104	37	15	3	11	21	46	70	33	9	5
	257	48	4	14	38	112	72	16	1	36	71	59	77	12	2	

* Endpoints read as marked inhibition at 24 hr, complete inhibition at 48 hr.

agents. The differences in sensitivity of the individual strains to the 3 analogues are summarized in Table II. About one-half of all the strains tested were equally sensitive to tetracycline and oxytetracycline and the remainder were distributed in a normal manner between those more sensitive and those more resistant; this was true both when the tests were read at 24 hours and again when they were read at 48 hours. The comparisons of the tests with tetracycline and those with chlortetracycline, however, reflected the deterioration of the latter during the course of the tests. This was manifested in the 24-hour readings by the large number of strains which required from 2 to 8 times as much chlortetracycline as compared with tetracycline to inhibit. This phenomenon was further exaggerated when the tests were read after 48 hours, at which time about 60% of the strains required the larger concentrations of chlortetracycline. In a number of instances, however, one of the analogues showed greater activity than the others against an appreciable proportion of strains of some species or genus. Thus, oxytetracycline appeared to be the most active against a large proportion of the strains of Pseudomonas; tetracycline appeared to be the most active against the few strains of Proteus that were tested, although high concentrations of all of the antibiotics were required to inhibit these strains. In the tests read at 24 hours chlortetracycline appeared to be the most active against a large proportion of the strains of Enterococcus and *Staphylococcus aureus*. The larger amounts of chortetracycline required to inhibit the strains of *Neisseria gonorrhoeae* and *N. meningitidis*, however, may be considered as reflecting the deterioration of that antibiotic in the course of the test. However, chlortetracycline appeared to be the least active against most of the strains of *E. coli* and *Shigella sonnei* even when the tests were read at 24 hours.

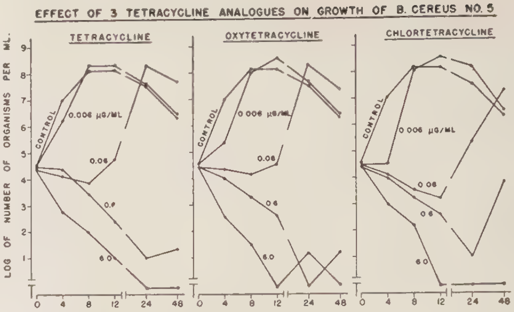

FIG. 1.

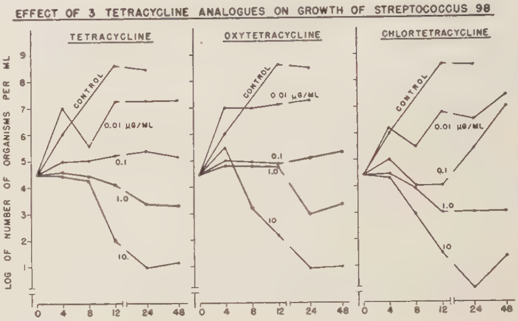

FIG. 2.

Growth curves. The effects of various concentrations of each of the 3 tetracyclines on the growth of *B. cereus* No. 5 and of *Streptococcus 98* are depicted in Fig. 1 and 2, respectively. The same inoculum (about 10^4 organisms/ml) was used in each instance and corresponded to that generally employed in this laboratory for antibiotic assays and in tests for sensitivity which employs serial dilutions of antibiotics in broth. The growth curves for each of the 3 antibiotics are very similar in the case of both organisms, the major difference being the earlier resumption of growth following inhibition in the presence of chlortetracycline in some instances. The effect of the size of the inoculum is shown for *B. cereus* in Fig. 3; the inocula used for the growth curves in the upper panels of this figure were 100 times greater than those shown in Fig. 1 and the inocula for the curves in the lower panel were such as to provide 30 to 60 organisms per ml. The tests with each inoculum and the 3 antibiotics were done at the same time, but those with the different inocula were done at different times. Tetracycline appeared to exert its inhibitory action for a longer period than did the other 2 analogues, but other differences among the antibiotics were relatively minor.

Discussion. The data presented indicate that, for the most part, the antibacterial action of each of the 3 tetracycline analogues is

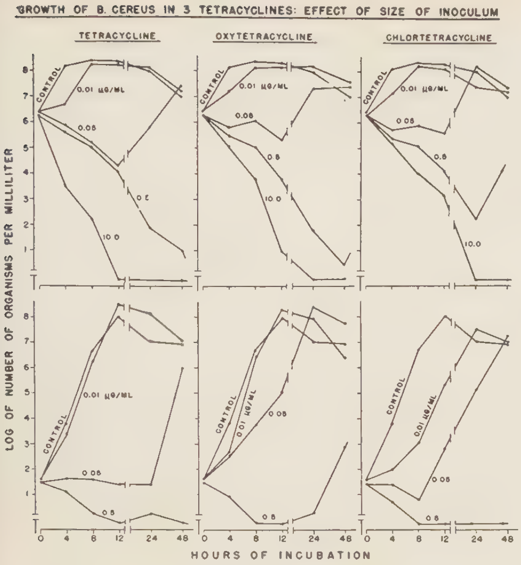

FIG. 3.

very similar under the conditions employed. In the case of individual strains, however, there were significant quantitative differences. The instability of chlortetracycline in the usual *in vitro* tests is well known. The effect of pH was studied and further differences were noted; these have varied somewhat when different organisms were used and the results are not presented since they require further study. In general all 3 agents were more active in an acid medium; this was particularly true of chlortetracycline which was the least active in an alkaline range, at which it was also the most unstable.

In the present study, recently isolated strains were used. This is important because of the increasing incidence of strains of certain organisms which are resistant to both oxytetracycline and chlortetracycline, presumably as a result of the wide use of these agents(5). In the tests for sensitivity, strains resistant to one of the tetracyclines were usually resistant in about the same general range to the other 2 analogues. In a separate study(6), a group of organisms made resistant *in vitro* by repeated subcultures in increasing concentrations of tetracycline and of oxytetracycline were subsequently tested and found to have acquired resistance to all 3 analogues to about the same degree.

Conclusions. The antibacterial action of tetracycline, oxytetracycline and chlortetracycline *in vitro* appeared to be very similar although significant quantitative differences are noted in the action of these 3 analogues on individual strains and under certain cultural conditions.

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1. Boothe, J. H., *et al.*, *J. Am. Chem. Soc.*, 1953, v75, 4621.
2. Conover, L. H., *et al.*, *ibid.*, 1953, v75, 4622.
3. Waller, C. W., *et al.*, *ibid.*, 1952, v74, 4981.
4. Stephens, C. R., *et al.*, *ibid.*, 1952, v74, 4976.
5. Finland, M., and Haight, T. H., *A. M. A. Arch. Int. Med.*, 1953, v91, 143.
6. Wright, S. S., and Finland, M., Proc. Soc. Exp. Biol. and Med., 1954, v85, 40.

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Effect of Total Body X-Irradiation on the Tryptophan Peroxidase Activity of Rat Liver. (20775)

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Knox has shown that the activity of tryptophan peroxidase-oxidase (TPO) of rat liver can be augmented *in vivo* by administration of large doses of the substrate, tryptophan, and to a lesser extent by injection of certain other amino acids, epinephrine, or histamine (1). The increase after tryptophan was considered to be an adaptive response, while the effect of non-specific compounds was attributed to pituitary-adrenal stimulation, since adrenalectomy inhibited the response to non-substrate compounds but not to tryptophan.

It was of interest to study the effects of total body x-irradiation on TPO of rat liver for several reasons. The conversion of tryptophan to formylkynurenine is a coupled two-step oxidation(2), the first step requiring peroxide and the second generating peroxide (which is used with high efficiency in the first step). Secondly an active catalase is necessary to the system to prevent accumulation of inhibitory concentrations of peroxide. Furthermore, free sulfhydryl groups seem to be necessary as inferred from inhibition of the reaction by arsenite. A final consideration was the role of the adrenals in response to irradiation (3), which raised the question whether any changes in activity of TPO produced by x-ray exposure were due directly to irradiation or were attributable to hormonal effects.

Methods. Most of the experiments utilized female Sprague-Dawley or Holtzman rats, 3 months old, weighing between 160 and 210 g; the 2 strains were identical in their response. Hypophysectomized Sprague-Dawley female rats were obtained commercially. Other operative procedures were carried out in the laboratory. Exposure to x-rays was at the rate of 225 r per minute from a machine operated at 250 kv, 15 ma, 0.5 mm Cu and 3.0 mm bakelite filters, 27 cm distance. A dose of 1000 r was used in most experiments. The method of assay for TPO was based on the formation of kynurenine measured spectrophotometrically at 360 mμ after zinc acetate-NaOH deproteinization of a reaction mixture containing homogenized liver, phosphate buffer, and tryptophan incubated at 38°C for 60 minutes(1). Under the conditions employed, kynurenine formation was linear in respect to time for 4 hours, and in respect to tissue concentration to at least 80 mg liver/ml reaction mixture. Measurement of ultraviolet absorption at other wave lengths indicated only negligible absorption attributable to compounds such as anthranilic acid, formylkynurenine, or kynurenic acid; the absence of other metabolites of tryptophan we further confirmed by paper chromatography(4).

Results. A nearly 2-fold increase in TPO activity was noted 4 hours after exposure of the rat to 1000 r. The activity declines gradually, reaching the normal range about 48 to 72 hours after exposure. Studies with varying amounts of radiation showed that the phenomenon was dose-dependent to at least 5000 r, although a linear relationship was not obtained. A significant increase was observable after doses as low as 200 r. Male rats responded as did females. Activation of TPO was also noted after x-irradiation of mice, but not of guinea pigs.

Incubation of mixed homogenates from livers of normal and irradiated rats showed in every case only single summation of activities, an indication that radiation neither destroyed an inhibitor nor created an activator of the system. Measurements of catalase and pyrogallol peroxidase showed no changes in the activities of these enzymes during the first 24 hours after irradiation.

Like the increase caused by epinephrine, histamine, etc.(1), the radiation-induced increase was inhibited by adrenalectomy (Table I). Animals maintained on either salt or cortical extract (Wilson; 0.2 ml/day) failed to show the characteristic response after exposure to 1000 r. Adrenal demedullation decreased the response somewhat but did not abolish it. Thyroidectomized rats did not differ from normals in respect to the activity of TPO after radiation.

Hypophysectomy led to variable results, depending upon the time elapsing between operation and sacrifice. Seven or 10 days after hypophysectomy the increase after x-ray was appreciable, although less than that seen in unoperated animals. The response of TPO became progressively less as the time intervals increased, due probably to atrophy of the adrenal cortex, since maintenance of hypophysectomized rats on 2 units/day of ACTH permitted a considerable restoration of the response in animals irradiated 14 days after operation. In no case, however, was the increase as marked in hypophysectomized rats as in the unoperated animals. A single injection of ACTH alone, even as much as 5 units, failed to produce a consistent increase in activity 4 to 6 hours after injection.

Attempts to inhibit the action of x-ray on TPO by administration of various drugs were not successful. Prior intraperitoneal or intravenous injection of large doses of atropine, physostigmine, tetraethylammonium chloride, procaine, cocaine, diphenhydramine, ephedrine, ascorbic acid, or cysteine produced *per se* a 200 to 300% increase in activity which

TABLE I. Effects of Total Body X-Irradiation and of Injection of Compound F and Tryptophan on Rat Liver Tryptophan Peroxidase.

Rats	Days after operation	Control No. rats	Control Avg*	Control σ	4 hr after 1000 r No. rats	4 hr after 1000 r Avg	4 hr after 1000 r σ	4 hr after 5 mg/kg Compound F No. rats	4 hr after 5 mg/kg Compound F Avg	4 hr after 5 mg/kg Compound F σ	2 g/kg dl-tryptophan 4 hr No. rats	2 g/kg dl-tryptophan 4 hr Avg	2 g/kg dl-tryptophan 4 hr σ	2 g/kg dl-tryptophan 6 hr No. rats	2 g/kg dl-tryptophan 6 hr Avg	2 g/kg dl-tryptophan 6 hr σ
Normal	—	48	7.7	1.5	16	13.9	2.4	6	15.5	3.4	5	66	9	9	121	18
Adrenalectomized	7- 9	20	7.4	1.9	11	7.0	1.3	4	17.1	2.4	4	60	13	Fatal within 6 hr		
Adrenal demedullated	7-14	7	8.6	1.2	6	10.1	.8	—	—	—				—	—	—
	21-28	13	8.1	1.5	13	11.4	1.1	4	12.1	2.3				7	74	25
Thyroidectomized	35	4	6.8	1.4	4	14.1	1.2	4	13.7	.5				4	81	14
Hypophysectomized	7	4	6.8	.1	4	9.6	.2									
	10	4	7.0	.3	4	9.1	.4									
	14	8	5.4	1.2	8	6.6	1.3	4	23.0	5.3				4	25.2	12.4
	28	4	6.3	.5	4	5.8	1.0									
Hypophysectomized + ACTH	14	4	6.8	.6	4	9.0	1.0	3	24.0	2.9				3	34.5	3.1

* Micromoles kynurenine formed/hr/g dry wt.

effectively masked the effect of radiation on TPO. Similarly, section of the spinal cord at C_6 resulted, after 24 hours, in a 5-fold increase in unexposed animals.

Intramuscular injection of 5 mg/kg of 11-hydroxy-17-corticosterone acetate (Compound F) in saline suspension was found to double the activity of TPO within 4 hours. Smaller amounts were proportionately less effective. This action of Compound F was not appreciably diminished by removal of the adrenal or thyroid, and was enhanced after hypophysectomy (Table I). Desoxycorticosterone acetate and tetrahydrocortisone had no stimulatory effect in adrenalectomized rats. Cortisone, on the other hand, acted like Compound F but was about ⅐ as effective. It may be pointed out further that neither Compound F nor x-ray exposure effected an increase in liver TPO activity of guinea pigs. The mechanism by which the corticosteroids act is as yet obscure; *in vitro* studies with rat liver slices incubated with Compound F and then homogenized and assayed for TPO activity have been inconclusive.

The 15-fold increase in activity of TPO resulting from injection of 2 g/kg of DL-tryptophan in normal rats (Table I) could be produced in irradiated animals until 4 days after exposure when the rats were manifestly moribund. In confirmation of Knox's findings (1), we found only a slight decrease in "adaptation" to tryptophan in adrenalectomized rats; we have also confirmed the high toxicity of tryptophan to adrenalectomized rats. Adrenal demedullation and thyroidectomy also failed to affect the response of TPO to tryptophan. In hypophysectomized rats, however, the injection of tryptophan produced much less effect than in normal rats (Table I).

Discussion. It is apparent that the increase in activity of the tryptophan peroxidase-oxidase system of rat liver observed after exposure to x-ray is not a direct consequence of radiation of the liver, but rather is secondary to an effect on the adrenals, since the increase does not occur in rats exposed to x-rays after adrenalectomy. Radiation is thus a nonspecific activator of the TPO system, resembling histamine, epinephrine, histidine, etc.(1). The proposed pituitary-adrenal mechanism of action of these non-specific activators (1) seems, however, to be open to question.

Summary. Total-body x-irradiation produces within a few hours a dose-dependent increase in the tryptophan peroxidase-oxidase system of rat liver. The increase does not occur in adrenalectomized rats, and hence cannot be construed as a direct effect of x-irradiation.

1. Knox, W. E., *Brit. J. Exp. Path.*, 1951, v32, 462.
2. Knox, W. E., and Mehler, A. H., *J. Biol. Chem.*, 1950, v187, 419.
3. Leblond, C. P., and Segal, G., *Am. J. Roentgenol.*, 1942, v47, 302.
4. Mason, M., and Berg, C. P., *J. Biol. Chem.*, 1951, v188, 783.

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Isolation and Chemical Composition of Complement-Fixing Antigens From Leptospires. (20776)

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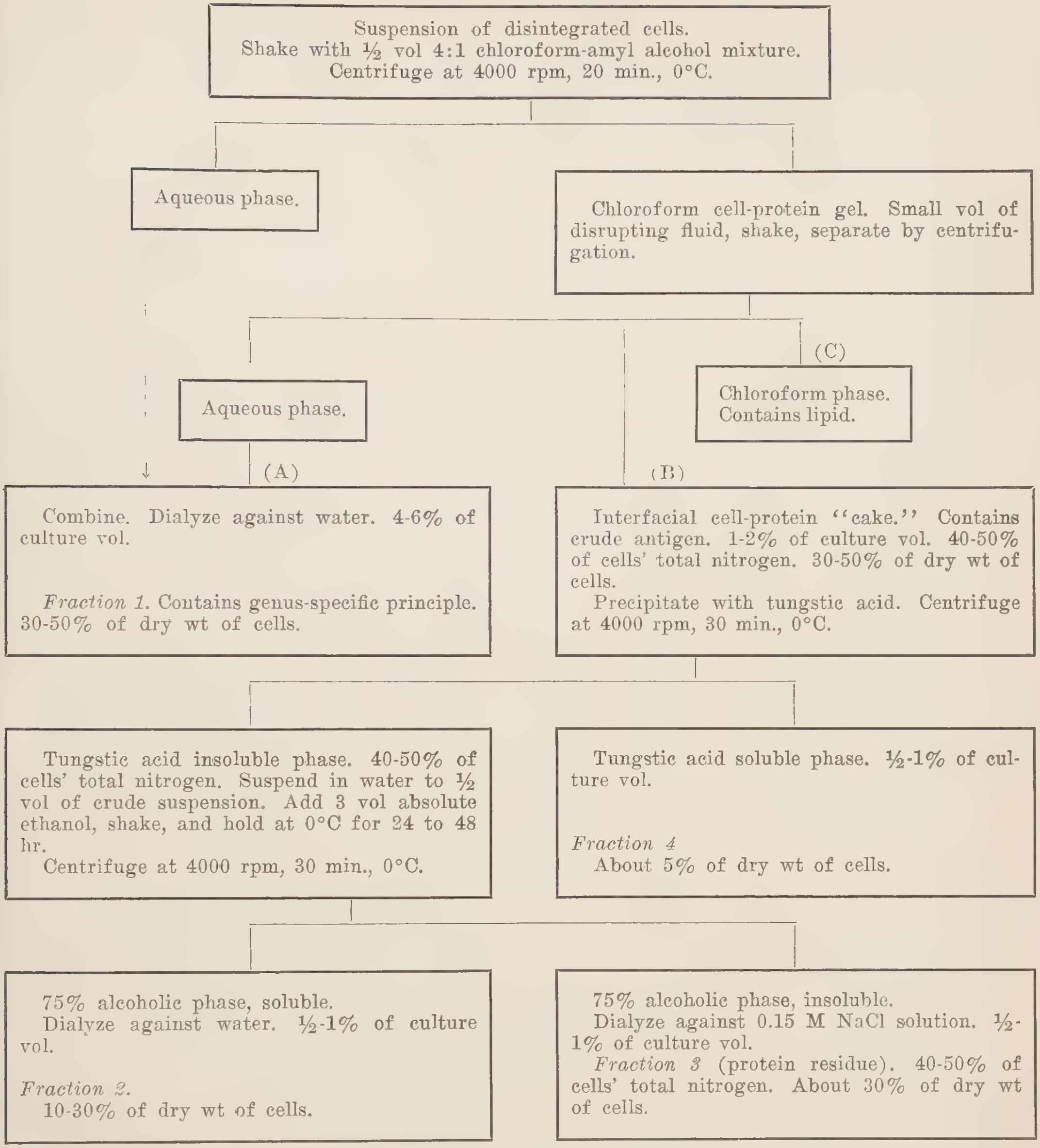
The purpose of this report is to present a comprehensive account of a fractionation procedure applied to the leptospiral cell and to show the comparative yield of the complement-fixing cell fractions from selected serotypes of leptospires. Fraction 1, a complement-fixing substance, has been isolated and its chemical and serological properties described(1-3).

The process of fractionation of the leptospiral cell has been extended to include the cellular debris remaining after extraction of Fraction 1. The cell residue served as the starting material from which were isolated three serologically active fractions.

Methods. Cultures of leptospires. Three serotypes of leptospires were employed, namely, *Leptospira icterohemorrhagiae* AB, *L. canicola* and *L. bataviae*. Leptospires were propagated and harvested as previously described(1,3). *Isolation of complement-fixing antigens.* The protocol for fractionation of the leptospiral cell is outlined in Fig. 1. Washed viable organisms were disrupted as previously described(1,3). After shaking with one-half volume 4:1 chloroform-n-amyl alcohol, the mixture was centrifuged at 4000 rpm for 20 minutes, at 0°C. The preparation separated into (A) an aqueous phase, (B) an interfacial cell-protein gel or chloroform "cake" and (C) a chloroform layer. The aqueous phase yielded, on further processing, a genus-specific antigen previously described (1-3). The cell protein debris (B) was separated from the chloroform layer, suspended in and thoroughly dialyzed against 0.15 M NaCl solution and disintegrated by sonic vibration. The final volume of this preparation was 1 to 2% of the original culture volume. The crude cell-protein antigen was fractionated as follows: The crude material was precipitated from its aqueous suspension by the addition of one-half volumes of each of 0.2 N H_2SO_4 and freshly prepared 2% sodium tungstate solution. A heavy flocculent precipitate appeared soon after the addition of tungstate solution. After 30 minutes, the acidified mixture (pH about 2.0) was centrifuged at low speed, in the cold, and yielded: 1) A soluble supernatant, which on subsequent dialysis against 0.15 M NaCl solution and pervaporation yielded a non-dialyzable, complement-fixing substance, identified as Fraction 4; 2) An insoluble sedimentable portion which was then suspended in water to one-half of the volume of the original crude suspension. Three volumes of absolute ethanol were added, the mixture shaken and held at about 0°C for 24 to 48 hours. After low speed centrifugation, the latter mixture separated into: a) A clear to slightly opalescent alcoholic phase which, after thorough dialysis against distilled water and pervaporation, yielded a non-dialyzable water-insoluble material designated Fraction



Suspension of disintegrated cells.
Shake with ½ vol 4:1 chloroform-amyl alcohol mixture.
Centrifuge at 4000 rpm, 20 min., 0°C.

Aqueous phase.

Chloroform cell-protein gel. Small vol of disrupting fluid, shake, separate by centrifugation.

Aqueous phase.

(C)

Chloroform phase. Contains lipid.

(A)

Combine. Dialyze against water. 4-6% of culture vol.

Fraction 1. Contains genus-specific principle. 30-50% of dry wt of cells.

(B)

Interfacial cell-protein "cake." Contains crude antigen. 1-2% of culture vol. 40-50% of cells' total nitrogen. 30-50% of dry wt of cells.

Precipitate with tungstic acid. Centrifuge at 4000 rpm, 30 min., 0°C.

Tungstic acid insoluble phase. 40-50% of cells' total nitrogen. Suspend in water to ½ vol of crude suspension. Add 3 vol absolute ethanol, shake, and hold at 0°C for 24 to 48 hr.

Centrifuge at 4000 rpm, 30 min., 0°C.

Tungstic acid soluble phase. ½-1% of culture vol.

Fraction 4

About 5% of dry wt of cells.

75% alcoholic phase, soluble.

Dialyze against water. ½-1% of culture vol.

Fraction 2.

10-30% of dry wt of cells.

75% alcoholic phase, insoluble.

Dialyze against 0.15 M NaCl solution. ½-1% of culture vol.

Fraction 3 (protein residue). 40-50% of cells' total nitrogen. About 30% of dry wt of cells.

FIG. 1. Fractionation of washed viable leptospires.

2; b) The 75% ethanol insoluble portion appeared as a packed white sediment. Suspended in and dialyzed against 0.15 M NaCl solution, the final product has been designated Fraction 3, or the protein residue antigen. The final volumes of each of the above fractions of the crude antigen were 0.5 to 1% of the original culture volume. The terminology Fractions 1, 2, 3 and 4 was arbitrarily applied to the complement-fixing fractions, representing, in the order indicated, decreasing amounts of serologically reactive material derived from the leptospiral cell. *Analytical procedures.* The application of analytical methods was the same as previously reported(1,3). Quantitative estimation of total carbohydrate was accomplished with an orcinol-sulfuric acid reagent(4). Ultraviolet absorption spectra were determined of reaction products of orcinol-sulfuric acid reagent with Fraction 1

TABLE I. Yield and Composition of Four Arbitrary Complement-Fixing Fractions from *L. icterohemorrhagiae* and *L. canicola.*

	L. icterohemorrhagiae 1	2	*L. canicola* 3
Fraction 1			
Yield (%)	43	19	39
Reactivity*	.10	.30	.30
% of total reactivity†	43	71	52
Composition (%)			
Nitrogen	6.2	8.8	9.5
Pentose	7.2	8.6	8.3
Desoxyhexose	5.9	6.0	4.1
DNA	8.9		12.1
Glucosamine	4.0		5.7
Phosphorus	1.0		1.1
Hexose	2.4		4.0
Total carbohydrate‡	26	32	26
Copper reducing substances§	21		
Fraction 2			
Yield (%)	12	24	29
Reactivity	.21	.07	.19
% of total reactivity	26	21	22
Composition (%)			
Nitrogen	3.8	2.8	.8
Pentose	4.8	2.7	2.7
Desoxyhexose		.3	Tr.
Glucosamine	Tr.		
Total carbohydrate	19	15	14
Fraction 3			
Yield (%)	30	23	28
Reactivity	.05	.01	.18
% of total reactivity	17	4	20
Composition (%)			
Nitrogen	10.8	8.6	11.4
Protein‖	(67.7)	(53.8)	(71.3)
Pentose	1.5	1.0	2.2
Fraction 4			
Yield (%)	4	1	10
Reactivity	.33	.21	.15
% of total reactivity	14	3	6
Composition (%)			
Nitrogen	2.8	4.4	2.2
Pentose	8.3	7.4	6.0
Desoxyhexose	5.3	6.0	4.1
Recovery			
% of organism	89	68	106

* Reactivity = 1/x, when x is minimal wt (μg) of material/tube yielding max. homologous hyperimmune serum titer.

† % of total activity = $\dfrac{\dfrac{\text{Yield (\%)}}{x}}{\Sigma\dfrac{\text{Yield (\%)}}{x}}$.

‡ Total carbohydrate as glucose, estimated by the orcinol-sulfuric acid procedure(4).

§ Copper reducing substances as glucose, after hydrolysis with 1N H_2SO_4 or 6N HCl, at 100°C, for 6-8 hr.

‖ ( ) Total nitrogen multiplied by 6.25.

Tr. = Trace.

and Fraction 2 antigens. The color development of sulfhydryl-sulfuric acid (CyR10) reagent(5) with Fraction 2 antigens was measured spectrophotometrically.

Results. The crude cell-protein antigen contained 30 to 50% of the dry weight and 40 to 50% of the nitrogen of the whole leptospiral organism. The active substance in the crude material was stable to heating at 100°C for 1 hour, sonic vibration, chloroform-amyl alcohol extraction, and precipitation with tungstic acid.

Relative yields and composition of the 4 fractions are shown in Table I. Calculations of serologically active substances were based on the minimal weight (μg) of each cell fraction which reacted with homologous hyperimmune rabbit serum at maximal titer (a dilution of 1:240 to 1:480 for *L. icterohemorrhagiae* and *L. bataviae,* respectively, and 1:120 to 1:240 for *L. canicola*). The yield of the complement-fixing fraction antigens of each of these serotypes of leptospires was 2- to 4-fold greater than that of the sonic-vibrated, lyophilized whole cell antigen of Randall *et al.*(6). The complement-fixing fractions were non-dialyzable materials, all of which contained pentose. *L. icterohemorrhagiae, L. canicola* and *L. bataviae* were each found to contain 4.4, 5.2 and 5.4% non-dialyzable pentoses by weight. Approximately 80% of the total pentose was recovered in Fractions 1 and 2. After acid hydrolysis Fraction 1 and 2 antigens gave positive ninhydrin reactions.

In the estimation of total carbohydrate, the orcinol-sulfuric acid method depends upon the absorption band at 425 mμ, a property characteristic of hexoses and pentoses. Fractions 1 and 2 illustrate this absorption band. Fraction 2 displays in addition a chemically unidentified maximum at 385-390 mμ (Fig. 2, A). Total carbohydrate, calculated as glucose, varied between 26 and 32% for three preparations of Fraction 1. After acid hydrolysis total carbohydrate was about the same as in unhydrolyzed materials. One preparation of Fraction 1 of *L. icterohemorrhagiae* contained 21% copper-reducing substance on hydrolysis. In addition to pentose and desoxyhexose(3), Fraction 1 contained glucosamine, hexose and other carbohydrate residues.

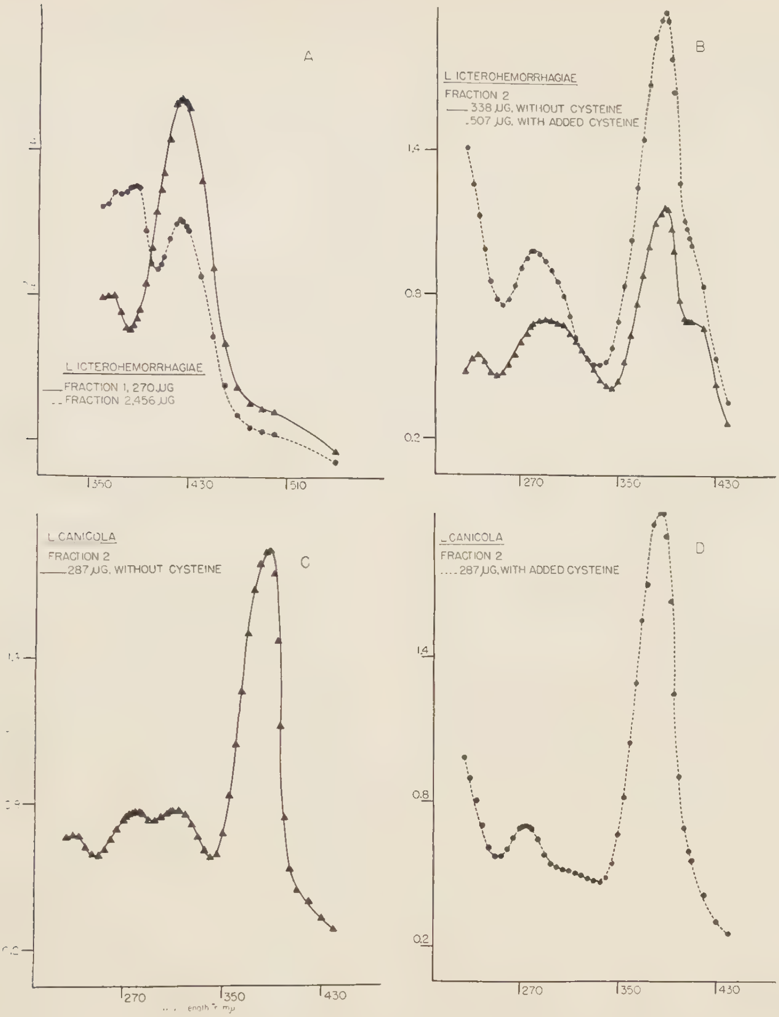


FIG. 2. A. Ultraviolet absorption spectra of materials after heating fractions 1 and 2 with an orcinol-sulfuric acid reagent(4), at concentrations of 270 and 456 µg, respectively, in 11 ml of reaction mixture. B, C, D. Absorption spectra of materials after heating fraction 2 antigens with sulfhydryl-sulfuric acid (CyR10) reagent(5). Spectra were measured on the Beckman DU spectrophotometer.

The chemical composition of Fraction 2 was distinguished from that of Fraction 1 by its lower content of nitrogen, pentose and desoxyhexose, by the apparent absence of glucosamine, and by the presence of an unidentified ultraviolet absorption peak at 385-390 mµ. It contained less total carbohydrate, as measured by the acid-orcinol procedure. At concentrations of 500 µg Fraction 2 reacted strongly with Dische's naphthol carbohydrate reagent and gave specific color complexes with sulfhydryl-sulfuric acid reagent(5). The absorption maxima of the cysteine-sulfuric acid color complexes with Fraction 2 lie approximately at the same wave lengths (Fig. 2, B, D). An absorption band was located at 265-275 mµ, and a second very sharp peak at 385-390 mµ. The latter peak occurred in the reaction mixture in the absence of cysteine (Fig. 2, B, C), but the extinction coefficient was slightly lower. On a weight basis, the (cysteine) extinction coefficient at 388 mµ was greatest for *L. canicola*, less for *L. icterohemorrhagiae* and least for *L. bataviae*. The active principle was insoluble in water at pH less than 7, and insoluble in absolute ethanol; but was readily soluble in water at pH greater than 7, and in 75% ethanol. Fraction 2 was isolated virtually free of protein only after denaturation of the crude cell-protein material with tungstic acid.

Extensive complement fixation tests have been applied to the crude cell-protein, intermediate products, and cell fraction antigens derived from the residue materials(7). The antigens of the leptospiral cell illustrated complement fixation reactivity with homologous hyperimmune rabbit serum at concentrations of antigen of 1 to 3 μg.

Discussion. A fractionation procedure has resulted in the isolation of 4 non-dialyzable, pentose-containing, serologically reactive fractions from the leptospire cell. A proportionately large amount of a complex, genus-specific complement-fixing antigen is isolated. A considerable quantity of crude cell-protein material remaining after removal of Fraction 1 is denatured with tungstic acid. The acid treated crude substance then serves as the starting material from which are isolated 3 additional cell fractions all of which show good complement fixation activity.

The active principle of Fraction 1 appears to be identified with the non-dialyzable pentose. Purification steps which result in the concentration of virtually all of the pentose polysaccharide moiety also retain virtually all of the complement-fixing reactivity(3). Preparations have been adsorbed with 20% Attaclay SF(8), resulting in the separation of the pentose polysaccharide moiety. The latter preparations illustrate reduced complement fixation activity depending on the amount of pentose separated.

Fractions 1 and 2 are chemically distinct compounds which are spectrophotometrically distinguishable from each other. Both preparations show relatively similar reactivity as complement fixation antigens. However, Fraction 2 contains about one-fourth of the amount of pentose found in Fraction 1. The pentose carbohydrate in Fraction 2 may be different. A possibility exists that the active principles in these fractions may be different. However, it has not yet been possible to determine the number of complement-fixing principles in the leptospire cell.

The cysteine-sulfuric acid (CyR10) reaction with Fraction 2 preparations of selected serotypes of leptospires indicates that this color complex contains an unidentified sharp peak at 385-390 mμ which may serve as a possible aid in the identification of the serotype.

Fraction 3 is predominantly protein, containing about 40% of the total nitrogen of the cell. Complement fixation reactivity may be accounted for by the pentose in this preparation, which is possibly a contaminant remaining after extraction of Fraction 2. It appears, therefore, that the protein residue is poorly reactive in the complement fixation test or has been seriously damaged during the fractionation procedure. Fraction 4 is a "non-protein-nitrogen" material and is apparently contaminated with carbohydrate residues found in Fraction 1.

Summary. A chemical separation procedure which yields four arbitrary cell fractions has been developed and applied to three serotypes of leptospires. The four complement-fixing fractions are non-dialyzable, pentose-containing materials, the serological activities of which have been measured semi-quantitatively. One or more complement-fixing principles, of nature not yet known, are concentrated in two chemically distinct, relatively free of protein, polysaccharide-containing cell fractions. The protein constituent (Fraction 3) of the leptospiral cell was poorly reactive in the complement fixation test. Spectrophotometric differences among preparations of Fraction 2 from three serotypes may prove to be an aid in the differentiation of serotypes.

The starting material and hyperimmune rabbit serums were provided by the Department of Veterinary Bacteriology. The complement fixation tests were carried out by the Department of Veterinary Clinical Pathology. The writer expresses his appreciation to Mr. William R. Clark and Dr. Marion E. Webster for glucosamine and reducing sugar, and hexose analyses, respectively.

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1. Schneider, M. D., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 655.

2. ———, *Bact. Proc., Soc. Am. Bact.*, 1953, p48.

3. ———, *J. Inf. Dis.*, in press.

4. Fernell, W. R., and King, H. K., *Analyst*, 1953, v78, 80.
5. Dische, Z., and Shettles, L. B., *J. Biol. Chem.*, 1948, v175, 595.
6. Randall, R., Wetmore, P. W., and Warner, A. R., *J. Lab. and Clin. Med.*, 1949, v34, 1411.
7. Unpublished data from this laboratory.
8. Hiatt, C. W., Rothstein, N., and Yager, R. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 703.

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Adrenal Response of Rats to Salicylamide and Sodium Salicylate with and Without Para-Aminobenzoic Acid.[*][†] (20777)

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The administration of either acetylsalicylic acid or sodium salicylate to rats has been shown to cause a marked drop in the content of ascorbic acid and to a lesser extent of cholesterol in the adrenals(1-4), an effect which is not found in the hypophysectomized animal(1,2). Cronheim *et al.*(1) gave salicylamide to a few animals and found its effect on the adrenals to be somewhat less than that of sodium salicylate. In later experiments these investigators administered para-aminobenzoic acid (PABA) along with sodium salicylate but failed to get an augmented effect(5). They suggested that their inability to demonstrate an additional effect with PABA was probably due to the fact that the animals were sacrificed too soon after the administration of the drug, namely, 2 hours.

Since the analgesic effect of salicylamide in the experimental animal has been shown to be greater than that of acetylsalicylic acid (6-8), it was decided to carry out a more extensive investigation on its adrenal effects, studying not only the intensity of its action but also the time required for the adrenal cholesterol and ascorbic acid to return to normal concentrations. For comparative purposes experiments were carried out with both salicylamide and sodium salicylate, alone or with an equivalent amount of PABA.

Methods. Albino rats were used throughout. All animals purchased from a commercial dealer were placed on our stock diet of Rockland rat pellets for at least a week before they were used in the experimental work. No food was allowed for 16 hours prior to the ad-

TABLE I. Effect of Oral Salicylamide with and without Para-Aminobenzoic Acid on Adrenal Cholesterol and Ascorbic Acid of Male Rats.

Concentration following drug administration, %				
Ascorbic acid		Cholesterol		
2 hr	4 hr	2 hr	4 hr	Remarks
.324 ± .053* (3)	.310 ± .037* (10)	3.25 ± .29* (3)	2.91 ± .37* (9)	Controls receiving gelatin
.164 ± .021 (4)	.274 ± .088 (14)	2.70 ± .61 (4)	2.51 ± .65 (14)	Salicylamide, 300 mg/kg
.180 ± .029 (4)	.272 ± .097 (15)	2.93 ± .43 (4)	2.51 ± .72 (15)	*Idem.* + equimolar quantity of PABA

* Stand. dev.
No. of animals in parentheses.

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[†] Part of this work was submitted by one of us, J. A. B., in partial fulfillment of requirements for the degree of Bachelor of Science, Randolph-Macon College.

TABLE II. Effect of Intraperitoneal Administration of Salicylamide or Sodium Salicylate with and without Sodium Para-Aminobenzoate on Cholesterol and Ascorbic Acid Content of Adrenals of Male Rats.

	Concentration following drug administration, %										
	Ascorbic acid					Cholesterol					
Exp.	2 hr	4 hr	6 hr	12 hr	24 hr	2 hr	4 hr	6 hr	12 hr	24 hr	Supplement
1	.422±.077* (10)	—	—	—	—	3.02± .44* (10)	—	—	—	—	Controls
	.202±.023 (4)	.238±.055 (8)	.254±.039 (8)	.385±.063 (11)	—	2.45± .23 (4)	2.08± .44 (8)	1.77±.59 (8)	3.11±.98 (11)		Salicylamide 300 mg, Na PABA 350 mg/kg
	.170±.024 (3)	.282±.034 (4)	.272±.083 (4)	.377±.062 (11)	—	2.28± .83 (3)	2.10± .39 (4)	1.80±.39 (4)	3.22±.81 (11)		Salicylamide 300 mg
2	.322±.077 (9)	—	—	—	—	3.36± .92 (9)	—	—	—		Controls
	.187±.024 (9)	.165±.030 (8)	.181±.020 (5)	.239±.024 (8)	.406±.031 (5)	2.53±1.55 (9)	2.46± .87 (8)	1.67±.34 (5)	2.03±.72 (8)	4.46±.40 (5)	Na salicylate 350 mg, Na PABA 350 mg/kg
	.178±.027 (9)	.158±.024 (7)	.191±.013 (5)	.254±.044 (8)	.351±.052 (5)	2.76± .56 (9)	2.15±1.03 (7)	1.67±.34 (5)	2.33±.43 (8)	3.64±.43 (5)	Na salicylate 350 mg

* Stand. dev. No. of animals in parentheses.

ministration of the drug. All animals were sacrificed by rapid decapitation. The adrenals were removed at once and analyzed for ascorbic acid by the method of Roe and Kuether(9) and for cholesterol by the method of Outhouse and Forbes(10). Animals raised in our own colony tended to have a higher concentration of both cholesterol and ascorbic acid in their adrenals than those purchased from commercial dealers. On account of this variation both test and control animals used in any one experiment were always of the same sex and of comparable age and stock. Data recorded in Tables I and II were obtained on rats purchased from the same dealer and weighing around 170 g at the time of sacrifice.

Table I shows the results obtained following the administration by stomach tube of 300 mg/kg of salicylamide with and without an equimolar amount of PABA. The salicylamide was suspended in .125% gelatin. Control animals received an equivalent volume of the gelatin solution. Previous work(11) showed that the insertion of the stomach tube was in itself a sufficient stress to cause a slight drop in the ascorbic acid content of the adrenals but that it did not exert a demonstrable effect upon the cholesterol concentration. The controls were sacrificed 2 and 4 hours after administration of the gelatin solution. It will be seen that the adrenal ascorbic acid concentration showed a marked drop 2 hours after the administration of the salicylamide, as compared with the controls, but was well on the way back to normal in 4 hours. Addition of PABA did not augment the salicylamide effect. The drop in adrenal cholesterol was much less definite than in ascorbic acid. The general trend, however, was distinctly downward. In other experiments in which the salicylamide dose was 500 mg/kg, the degree of reduction of ascorbic acid was about the same as was obtained with the 300 mg/kg dose but the effect on the cholesterol concentration was somewhat more definite.

In an attempt to obtain more definite information on the adrenal response to salicylamide and the possible influence of PABA, it was decided to study their effect when given intraperitoneally, using the sodium salt of

PABA instead of the free acid. Salicylamide was given at a level of 300 mg/kg and Na-PABA at a level of 350 mg/kg. Each animal received 2 ml of the appropriate solution per 100 g of body weight. When salicylamide was used it was suspended in .125% gelatin-.64% NaCl solution. The NaCl was omitted when Na-PABA was used. It was also omitted in the accompanying experiments in which Na-salicylate was used. Control animals received 2 ml of the gelatin-NaCl solution per 100 g of body weight. A few were given 350 mg/kg of Na-PABA only, and since the results on these animals did not differ from those obtained in the other controls, they have been included in the control values.

The experimental results which are given in Table II show that the addition of the Na-PABA did not affect the adrenal changes, either from the standpoint of intensity of effect or duration of action. Although the drop in both the ascorbic acid and cholesterol was very much the same with both salicylamide and Na-salicylate, the duration of the effect was somewhat longer with Na-salicylate. Both compounds showed the lowest average concentration of adrenal cholesterol 6 hours after their administration, but the return to normal was more rapid in the salicylamide-treated animals. The lowest concentration of adrenal ascorbic acid was found in those animals sacrificed 2 hours after salicylamide, but 4 hours following Na-salicylate administration. The ascorbic acid content of the adrenals of the salicylamide-injected animals was showing a distinct return towards normal by the 4th hour, the time at which the Na-salicylate-injected animals showed the lowest values.

Discussion. The concentration of both ascorbic acid and cholesterol in the adrenals at a given time must represent a balance between their rates of utilization or removal on the one hand, and their rates of formation or deposition on the other. Consequently, the more prolonged effect of Na-salicylate on the concentration of these substances in the adrenal does not necessarily signify a longer period of increased adrenal activity. Further studies along other lines would be necessary in order to establish a definite answer.

Summary. Both oral and intraperitoneal administration of salicylamide to rats was found to cause a drop in the ascorbic acid and cholesterol content of the adrenal glands. The simultaneous administration of PABA or its sodium salt along with either salicylamide or Na-salicylate had no influence on the intensity or duration of the adrenal effect.

---

1. Cronheim, G., King, J. S., and Hyder, N., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 51.
2. Hetzel, B. S., and Hine, D. C., *Lancet*, 1951, v261, 94.
3. Van Cauwenberge, H., *ibid.*, 1951, v261, 374.
4. Blanchard, K. C., Dearborn, E. H., Maren, T. H., and Marshall, E. K., Jr. *Bull. Johns Hopkins Hosp.*, 1950, v86, 83.
5. Cronheim, G., King, J. S., and Hyder, N., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 109.
6. Hart, E. R., *J. Pharm. Exp. Therap.*, 1947, v89, 205.
7. Ichniowski, C. T., and Hueper, W. C., *J. Am. Pharm. Assoc., Sci. Ed.*, 1946, v35, 225.
8. Litter, M., Moreno, A. R., and Donin, L., *J. Pharm. Exp. Therap.*, 1951, v101, 119.
9. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, v147, 399.
10. Outhouse, E. L., and Forbes, J. C., *J. Lab. Clin. Med.*, 1940, v25, 1157.
11. Forbes, J. C., and Duncan, G. M., *Quart. J. Stud. Alc.*, 1951, v12, 355.

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Cross-Resistance Among 3 Tetracyclines.* (20778)

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In a previous paper(1) it was shown that the 3 chemically closely related antibiotics tetracycline, oxytetracycline (Terramycin) and chlortetracycline (Aureomycin) exerted very similar antibacterial action against most of the pathogenic strains which were studied. In general, the bacteria tested were equally sensitive or resistant to all 3 analogues under the conditions of the test that was used, although for individual strains or species there was a significant quantitative difference in the action of one or another of these analogues. Earlier studies(2-7) had shown considerable cross-resistance between chlortetracycline and oxytetracycline and some cross-resistance between these 2 agents and chloramphenicol. It was, therefore, of interest to determine whether similar cross-resistance could be demonstrated *in vitro* with the new antibiotic, tetracycline, which lacks both the organic chlorine ion of chlortetracycline and the OH group which characterizes oxytetracycline.

Materials and methods. Tetracycline (Achromycin) hydrochloride was provided in crystalline form in sterile vials by Dr. Stanton M. Hardy of Lederle Laboratories. The other antibiotics employed were similar to those used in previous studies(8). The organisms used were recently isolated from single colony cultures of infectious material from patients in the hospital. These were grown in brain-heart infusion broth (Difco), pH ± 7.4, containing serial 4-fold dilutions of tetracycline and of oxytetracycline, and subcultures were made at 3- or 4-day intervals from the tube containing the highest concentration of antibiotic which permitted nearly optimum growth to another series of tubes of broth containing the same antibiotic. (Chlortetracycline was not used for such subcultures because during such long periods of incubation deterioration of that antibiotic is so great as to render the results invalid for comparisons.) After 20 such subcultures, the 2 series of strains from the antibiotics and their parent strains which had not been exposed to any antibiotics, were tested simultaneously to each of the 3 tetracycline analogues by a 2-fold agar plate dilution method(9). All of the organisms were then subcultured once in antibiotic-free broth and strains from all 3 series were tested simultaneously for sensitivity to penicillin, streptomycin, chloramphenicol, bacitracin, polymyxin B (Aerosporin), neomycin and erythromycin by the same agar plate dilution method. The sensitivity of any strain was considered to be the minimum concentration of the antibiotic which failed to produce visible growth in 24 hours.

Results. Cross-resistance among the tetracyclines. The sensitivities to tetracycline, oxytetracycline, and chlortetracycline of the parent strains and of those which had been subcultured 20 times in broth containing graded concentrations of either tetracycline or oxytetracycline are listed in Table I. Each of the organisms increased in resistance from 16- to 256-fold against the antibiotic to which it had been exposed. All of these organisms also increased in resistance against each of the other 2 analogues, and the increases were of the same order of magnitude as against the homologous antibiotics; in some instances the increases in resistance against the heterologous agent exceeded those which were demonstrated against the antibiotic to which the organisms had been exposed, but the reverse was observed just as frequently.

Cross-resistance to other antibiotics. Tests with the 3 series of cultures showed no significant differences in sensitivity to penicillin, streptomycin, bacitracin, polymyxin B, neomycin or erythromycin; in every instance the minimum inhibiting concentration of the tetracycline-resistant and oxytetracycline-resistant variants for each of these antibiotics was

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TABLE I. Cross Resistance to Other Tetracycline Analogues after Repeated Subcultures in Broth Containing Tetracycline or Oxytetracycline.

Strain	Minimum inhibiting concentration, μg/ml								
	Tetracycline			Oxytetracycline			Chlortetracycline		
	P*	T×20	O×20	P	T×20	O×20	P	T×20	O×20
K. pneumoniae	6.3	800	800	6.3	800	800	25	400	400
Enterococcus 1	1.6	50	800	3.1	100	800	3.1	50	100
" 2	1.6	50	800	3.1	100	800	3.1	50	100
Staph. aureus 1	.8	25	100	.8	25	50	1.6	25	25
" " 2	.8	100	25	1.6	200	50	1.6	50	12.5
E. coli	3.1	400	50	3.1	200	50	12.5	200	100
A. aerogenes	6.3	800	800	6.3	800	800	12.5	200	200
Strep. viridans	1.6	100	50	.8	100	200	1.6	50	50

* P = parent strain not previously exposed to antibiotics; T×20 = same strain after 20 subcultures in broth containing tetracycline; O×20 = same strain after 20 subcultures in broth containing oxytetracycline.

TABLE II. Cross-Resistance to Chloramphenicol in Organisms Exposed to Tetracycline or Oxytetracycline.

Organism	M.I.C.* of chloramphenicol, μg/ml		
		Strain subcultured 20 times in	
	Parent strain	Tetra-cycline	Oxytetra-cycline
K. pneumoniae	50	>400	>400
Enterococcus 1	25	50	25
" 2	25	25	25
Staph. aureus 1	25	50	25
" " 2	25	100	25
E. coli	25	400	400
A. aerogenes	100	>400	>400
Strep. viridans	6.3	25	25

* Minimum inhibiting concentration.

either identical with that of the parent strain or varied from it by only a single 2-fold dilution. The sensitivities to chloramphenicol, however, showed significant changes and are listed in Table II. As in the previous study with chlortetracycline and oxytetracycline(7), only the coliform organisms which had been made resistant to the tetracyclines exhibited significant cross-resistance to chloramphenicol, whereas the tetracycline- and oxytetracycline-resistant gram-positive organisms showed either no cross-resistance or only slight increases in resistance to chloramphenicol.

Miscellaneous observations. The strains of the 20th transfer in the antibiotics were also compared in several ways with their respective parent strains. On antibiotic-free blood agar plates, the strains transferred directly from the antibiotic containing broth grew more slowly and formed much smaller colonies. This was particularly striking with the staphylococci of which there were tiny colonies, visible only after magnification, interspersed among somewhat larger colonies; the small ones corresponded morphologically to the so-called G forms(10). These staphylococcal colonies also produced less pigment than the parent strain. The strain of *Staph. aureus* 2 which had been subcultured in oxytetracycline failed to coagulate human plasma, whereas all of the other staphylococcal strains did so when tested at the same time with the same plasma. Penicillinase was produced by both the parent and the resistant variants of *Staph. aureus* 2, when the latter were tested after a single subculture in antibiotic-free broth. On eosin-methylene blue agar, the 3 coliform organisms that had been subcultured in the antibiotics produced smaller colonies than their respective parent strains, and the colonies of the antibiotic-resistant variants of *K. pneumoniae* were flatter and less mucoid than those of their parent strain. On gram stain the organisms taken directly from the antibiotic-containing broth were generally smaller and much more pleomorphic than their respective parent cultures grown in the same broth without antibiotic. The resistant gram-positive organisms stained poorly and the resistant variants of *K. pneumoniae* had lost their capsules. Resistance to heat was retained by all the strains of enterococci. The reaction of *E. coli* and *A. aerogenes* to Simmons citrate agar was unchanged.

Conclusions. Organisms made resistant *in vitro* to either tetracycline or oxytetracycline by repeated subcultures in these antibiotics developed essentially complete cross-resistance to each other and to chlortetracycline. No cross-resistance or increases in sensitivity to penicillin, streptomycin, bacitracin, polymyxin, neomycin or erythromycin developed as a result of the increases in resistance to the tetracyclines. Three strains of coliform bacilli each showed significant cross-resistance to chloramphenicol following subcultures in either tetracycline or oxytetracycline, but similar cross-resistance was not observed in 5 gram-positive coccal strains.

1. Love, B. D., Jr., Wright, S. S., Purcell, E. M., Mou, T. W., and Finland, M., Proc. Soc. Exp. Biol. and Med., 1954, v85, 25.

2. Pansy, F. E., Khan, P., Pagano, J. F., and Donovick, R., Proc. Soc. Exp. Biol. and Med., 1950, v75, 618.

3. Kaipainen, W. J., Does Induced Resistance of Bacteria to One Antibiotic Result in Simultaneous Sensitivity Changes to the Other Antibiotics? Suppl. 1, v29, *Ann. Med. Exp. Biol. Fenniae*, 1951, (Helsinki), 96 pp.

4. Fusillo, M. H., and Romansky, M. J., *Antibiot. and Chemother.*, 1951, v1, 107.

5. Monnier, J. J., and Schoenbach, E. B., *Antibiot. and Chemother.*, 1951, v1, 472.

6. Gezon, H. M., and Fasan, D. M., *Science*, 1951, v114, 422.

7. Gocke, T. M., and Finland, M., *J. Lab. and Clin. Med.*, 1951, v38, 719.

8. Haight, T. H., and Finland, M., *ibid.*, 1952, v39, 637.

9. Jackson, G. G., and Finland, M., *A. M. A. Arch. Int. Med.*, 1951, v88, 446.

10. Wise, R. I., and Spink, W. W., *J. Clin. Invest.*, 1953, v32, 612.

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Prevention of Urinary Calculi Formation in Mink by Alteration of Urinary pH.* (20779)

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Chemical analyses of mink urinary calculi (1) have shown that the main component is magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4 \cdot 6H_2O$). Small amounts of calcium phosphate and magnesium phosphate may also be present. Magnesium ammonium phosphate calculi are a type of calculi known to form in an alkaline urine. *In vitro* studies(2) have shown that magnesium ammonium phosphate is quite soluble in water when the pH is reduced to 6.0 or less. However, there is a sharp drop in the solubility of this compound when the pH range is 6.0 to 7.5. The pH of urine from mink on ranch diets varies from 5.5 to 7.5(3,4). It appears that the pH of the urine is an important factor in the formation of urinary calculi in mink.

The alteration of urinary pH by dietary means may be a logical method to attack the urinary calculi disease problem in mink. A practical and inexpensive means of acidifying the urine is the addition of the chemical ammonium chloride (NH_4Cl) to the diet. Studies on the use of ammonium chloride for the prevention of urinary calculi in mink are presented in this report.

Experimental. Mink were placed in individual metabolism cages and were given the following ranch diet: commercial mink cereal,† 25%; horsemeat (with bone), 60%; and horse liver, 15%. During the ammonium chloride feeding period (Experimental Period

TABLE I. Alteration of Mink Urinary pH by Ammonium Chloride.

Exp. period	Diet	Urinary pH* Avg	Urinary pH* Range
I	Ranch diet	6.4	6.0–6.8
II	Ranch diet + 1 g NH_4Cl/mink/day	5.7	5.5–5.9

* Avg of 5 different 24-hr urine collections from 17 mink.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

† Federal Xtra Mink Mix.

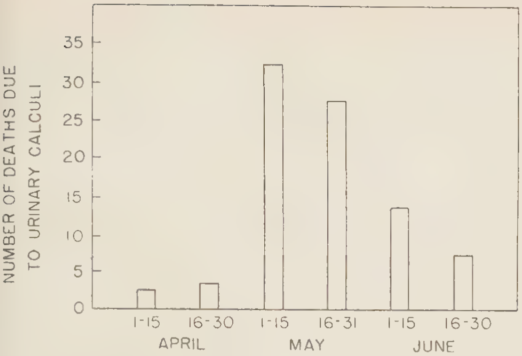

FIG. 1. Typical losses of pregnant mink due to urinary calculi on a mink ranch during the spring of 1952.

II) 1 g of ammonium chloride (U.S.P. grade) was added to each mink's daily ration with thorough mixing. The mink urine samples were collected under toluene. Immediately after each 24-hour urine collection period the pH of the urine was measured with a glass electrode pH meter.

The *results* are shown in Table I. The addition of ammonium chloride at a level of 1 g per mink per day lowers the average pH of the urine from 6.4 to 5.7. With ammonium chloride in the diet the range of urinary pH among individual animals is from 5.5 to 5.9.

The incidence of urinary calculi in mink is largely confined to two distinct periods of the year. In the spring calculi are found primarily in female mink during pregnancy or soon after whelping of the kits. In the summer months calculi occur mainly in young male kits. Fig. 1 shows typical losses of pregnant mink due to urinary calculi on a mink ranch during the spring of 1952. Within a period of 3 months 84 out of the 2,000 pregnant mink on the ranch were lost with urinary calculi. From Fig. 1 it is seen that the critical period of urinary calculi formation in female mink is during the months of April, May and June. The acidification of mink urine during this critical period by dietary ammonium chloride might be a means of preventing urinary calculi formation in the mink.

An experiment designed to study the effect of dietary ammonium chloride on the formation of urinary calculi in mink was set up in the spring of 1952. Six hundred Royal Pastel female mink (bred in March) were divided into 2 experimental groups. The 400 mink in Group I received the regular ranch diet. The 200 mink in Group II received the regular ranch diet plus 1 g of ammonium chloride per mink per day (200 g of ammonium chloride added daily to their portion of the ranch ration). The ammonium chloride was added to the diet of Group II from April 22 to May 27. From the data shown in Table II it is evident that the addition of ammonium chloride to the mink's diet at a level of 1 g per mink per day will prevent the formation of urinary calculi. By July 1, 16 out of the 400 mink in Group I on the ranch diet had died with urinary calculi. Of the 200 mink in Group II on the ranch diet plus ammonium chloride, only one mink had died with urinary calculi. This mink died only 3 days after the beginning of the experiment. This would indicate that the calculi had already been formed in the bladder of the mink. There were no indications of toxicity of ammonium chloride to the mink.

In the spring of 1953 the use of ammonium chloride for the prevention of urinary calculi in mink was extended to include over 50,000 mink on 100 mink ranches in the United States and Canada. In order to determine the minimum level of ammonium chloride in the diet required to prevent the formation of urinary calculi, a ½ g level as well as the 1 g level of the chemical was tested. Preliminary pH studies (6 mink) had shown that a level of ½ g of ammonium chloride per mink per day would lower the urinary pH from 6.3 to 5.8. Ten thousand mink received ammonium chloride at a level of 1 g per mink per day and 40,000 mink received the chemical at a level of ½ g per mink per day. Because it

TABLE II. Effect of Dietary Ammonium Chloride on Formation of Urinary Calculi in Mink.

Exp. group	No. of mink	Diet	No. of deaths due to urinary calculi
I	400	Ranch diet	16
II	200	Ranch diet + 1 g NH_4Cl/mink/day	1*

* This mink died 3 days after start of exp.

was impractical for the mink ranchers to divide their mink into separate groups for control measures, whole ranches were placed on the ammonium chloride feeding program. The experimental period was from April 10 to June 1, 1953. Mink ranches feeding ammonium chloride at the 1 g level had small losses of mink with urinary calculi (less than .2%). Over half of those ranches which fed the ½ g level of the chemical had some significant losses of mink with urinary calculi (.5-3.0%).

Discussion. The addition of ammonium chloride to the diet of the mink at a level of 1 g per mink per day is a practical and effective means of preventing the formation of urinary calculi in mink. The feeding of ammonium chloride at a level of ½ g per mink per day has been found to be ineffective in preventing urinary calculi formation on many mink ranches. It should be emphasized that the ammonium chloride regimen for the control of urinary calculi disease in mink is only a prophylactic measure. The urinary pH produced by the recommended level of ammonium chloride feeding is not low enough for rapid dissolution of the mink calculi. Although small urinary calculi may be dissolved, the animal may die before the larger calculi have been dissolved by the slightly acid urine. Partially dissolved calculi have been observed in mink from mink ranches starting the ammonium chloride feeding program late in May, *i.e.*, towards the end of the critical period of urinary calculi formation in mink.

The effectiveness of the ammonium chloride feeding program in preventing calculi formation in mink emphasizes the importance of urinary pH in urinary calculi formation. Urinary pH may be altered by dietary as well as by chemical means. It may be possible to control urinary calculi disease in mink by dietary management. The low incidence of urinary calculi on ranches feeding high levels of fish would indicate that control of urinary calculi in mink by dietary management may be feasible.

Summary. The formation of magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6H_2O$) urinary calculi in mink can be prevented by the addition to the mink's diet of 1 g of ammonium chloride (NH_4Cl) per mink per day. A level of ½ g of NH_4Cl per mink per day has been found to be ineffective in preventing urinary calculi formation in mink.

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1. Leoschke, W. L., Zikria, E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 291.
2. Vermeulen, C. W., Grove, W. J., Goetz, R., Ragins, H. D., and Correll, N. O., *J. Urology*, 1950, v64, 541.
3. Kubin, R., and Mason, M. M., *Cornell Vet.*, 1948, v38, 79.
4. Leoschke, W. L., and Elvehjem, C. A., unpublished data, 1953.

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Localization of Cu^{64} in Serum Fractions Following Oral Administration: An Alteration in Wilson's Disease. (20780)

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Previous work(1,2) has shown that the circulating serum iron is bound specifically to a β_1 globulin and that under normal conditions no binding occurs with any of the other plasma components. Experiments *in vitro*, however, have shown that this metal combining globulin will also form complexes with copper and zinc(2,3), and it was initially suggested that copper may be transported in the circulation attached to the β_1 metal combining globulin. More recently Holmberg and Laurell(4) have shown that copper in human serum is largely bound to a protein with a mobility of an α_2 globulin. This protein has been named ceruloplasmin and has been shown to exhibit oxidase activity towards a variety of substrates, the best of which is paraphenylenediamine(5,6). A small percentage of the serum copper, however, does not exhibit oxidase activity, and it has been suggested that this copper represents transport copper(7). The precise serum fraction to which the non ceruloplasmin copper is bound and the form in which it is transported remains unknown.

In this report electrophoretic separation of the serum of man and the rat following the oral administration of radioactive Cu^{64} has enabled a more precise analysis of the roles of ceruloplasmin and non ceruloplasmin copper to be undertaken. Patients with Wilson's disease have also been studied since this disease is characterized by a deficiency of ceruloplasmin(8,9) associated with an increased urinary excretion of copper(10). Patients with cirrhosis of the liver exhibiting an increased ceruloplasmin level(11) were also studied.

Methods and materials. Radioactive copper was obtained from the Oak Ridge National Laboratory in the form of copper wire. This was converted to copper nitrate, and was administered orally(12). The rats received 2 mc containing 25.6 mg copper and in man a dose of 1 mc containing 12.8 mg copper was used. Samples of blood were removed at timed intervals and radioactivity measurements carried out using a Geiger Müller counter. Electrophoretic separation of 1-4 ml samples of serum was carried out in a starch supporting medium by a slight modification of the method described by Kunkel and Slater(13). To eliminate air spaces between the starch block and the material covering the block, wax paper was applied to the starch while still wet following pouring of the starch buffer mixture into a mold. The starch block was then dried by means of blotting paper at the ends and sides. The close contact of the wax paper to the starch block prevented condensation of liquid in the form of small bubbles beneath the wax paper during the electrophoresis experiment. The sample was applied through a flap in the wax paper. After the separation, the starch block was cut in segments and the liquid removed from each segment by displacement filtration(13). Aliquots were taken for measurements of radioactivity and also for the construction of a protein curve using the modified Folin Tyrosine method(14).

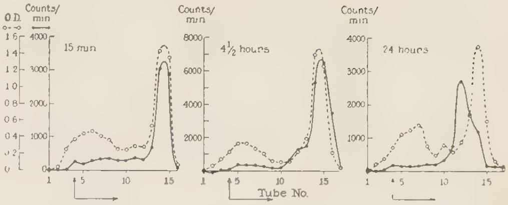

FIG. 1. Electrophoretic separation of rat serum following the administration of Cu^{64} by mouth. In this and succeeding 2 figures the solid line represents radioactivity and the dashed line represents the protein curve.

Results. Observations on rats. Cu^{64} was added *in vitro* to rat serum which was then subjected to zone electrophoresis in a starch supporting medium. When 2.0 μg copper was added to the serum, it was found that the radioactivity was confined to the albumin component. The radioactive copper was also given orally to rats who were bled at timed intervals, and the serum obtained subjected to zone electrophoresis. Fifteen minutes after its administration, the radioactive copper was located primarily in the albumin fraction. At 4½ hours the majority of the radioactivity was still present in the albumin but a hump had occurred in the α_1 globulin region accounting for about 15% of the total activity in the serum. At 24 hours the albumin peak had decreased strikingly and over 70% of the total activity was now found in the α_1 globulin region. These results are graphically illustrated in Fig. 1. The close proximity of the globulin peak to the albumin peak made complete separation from the albumin peak difficult. However, the peak of maximum radioactivity at 24 hours was well separated from the albumin peak. The slight hump on the descending curve of the α_1 globulin copper peak represents, at least in part, copper remaining in the albumin fraction. Direct measurements of oxidase activity in rat serum employing paraphenylenediamine as the substrate localized all the ceruloplasmin activity to the α_1 globulin region. No oxidase activity was demonstrated in the albumin peak.

Observations in man. Experiments were carried out in control subjects, patients with cirrhosis of the liver and patients with Wilson's disease. Cu^{64} was added *in vitro* to normal serum, serum from patients with cir-

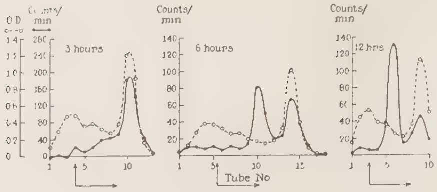

FIG. 2. Distribution of radioactive copper in the electrophoretically separated fractions of the serum of control subjects following the administration of Cu^{64} by mouth.

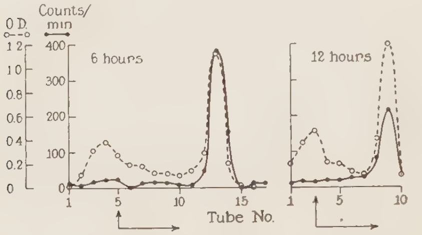

FIG. 3. Distribution of radioactive copper in the electrophoretically separated fractions of the serum from a patient with Wilson's disease following oral administration of Cu^{64}.

rhosis of the liver and serum from 2 patients suffering from Wilson's disease. Starch zone electrophoresis revealed that in all the groups studied when 0.25 μg copper was added to the serum the added copper became bound to the albumin fraction since all the radioactivity was confined to this component. Small amounts of radioactive copper appeared in other fractions when 2.5 μg copper was added to 1 cc of serum. In the *in vivo* experiments the low radioactivity found in the serum made it necessary to separate at least 2 ml serum.

Blood samples drawn 15 minutes after the oral administration of 1 mc of Cu^{64} showed that the radioactivity was again confined to the albumin peak. At 6 hours, in control subjects, a significant proportion of the radioactivity had been transferred to the α_2 globulin region (Fig. 2). In patients with Wilson's disease, however, the radioactivity was still confined to the albumin peak, and no radioactivity was present in the α_2 globulin fractions (Fig. 3). In control subjects blood drawn at 12 hours revealed that over 70% of the radioactivity in the serum was now located in the α_2 globulin fraction. In patients with Wilson's disease, however, the majority of the radioactivity was still confined to the albumin and the α_2 globulin fraction of the serum contained little or no radioactive material. The short half life of copper prevented long term studies being carried out, but serum drawn at 24 hours, in patients with Wilson's disease, still showed a considerable amount of radioactive copper associated with the albumin fraction with little or none in the ceruloplasmin. In normal subjects at 24 hours the major peak was now in the ceruloplasmin and only a very small amount of radioactive copper was still associated with the albumin component.

Serum from normal subjects who had not received radioactive copper, was also subjected to zone electrophoresis and confirmed that the majority of the serum copper was located in the α_2 globulin fraction (Fig. 4). A small peak present in the albumin fraction was due in part to contamination with trace amounts of copper since the height of this peak was dependent in large part on the care taken to eliminate contaminating copper from the starch and buffer materials(15). Moreover, over 90% of the total copper in the serum could be accounted for by the copper in the α_2 globulin peak. All the oxidase activity of the serum was confined to the α_2 globulin peak which therefore represents ceruloplasmin(15). The ceruloplasmin copper peak in rat serum migrates closer to the albumin than in the human and the oxidase activity is also located in this fraction.

An additional alteration noted in the patients with Wilson's disease following the oral administration of Cu^{64} was an increased urin-

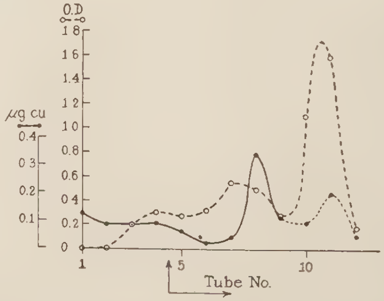

FIG. 4. Curves showing the distribution of chemically determined copper in normal serum. Ceruloplasmin, measured enzymatically, is localized in Tube 8.

ary excretion of Cu^{64} compared with normal subjects. This was also observed after intravenous administration. Fecal excretion of Cu^{64} was however diminished compared with normal subjects when the copper was given by the latter route.

Discussion. It has been shown by Klotz (16) that serum albumin is capable of binding copper *in vitro*. The results of this study show that under normal conditions copper added to serum is not bound to the metal combining globulin but is bound to the albumin fraction of serum. The possibility that some other protein migrating with the albumin fraction binds the copper appears remote. Following the oral administration of copper to rats, normal subjects and patients with Wilson's disease the copper initially is also bound to the serum albumin fraction. In normal subjects and patients with cirrhosis of the liver the copper appears relatively quickly in the ceruloplasmin and disappears from the albumin. In the rat the shift of the radioactivity to the α_1 globulin region was slower than that found in control subjects and is probably related to the difference in the amount of copper administered to the two groups. In patients with Wilson's disease, however, there is a specific deficiency of ceruloplasmin and the copper remains confined to the albumin component and no shift of the radioactive copper to other plasma components occurs. Earl and Selverstone (17) have used 50% precipitation with ammonium sulfate to fractionate the serum proteins following the oral administration of Cu^{64}. In normal subjects and patients with Wilson's disease the radioactivity first appeared largely in the supernatant. Later the radioactivity in normal subjects was found in the precipitate, whereas in patients with Wilson's disease the radioactivity still largely remained confined to the supernatant. The present work suggests that the serum component which is not precipitated by 50% ammonium sulfate and to which radioactive copper is bound in Wilson's disease is serum albumin, and no significant binding with any other plasma component occurs.

Since copper is first associated with the albumin fraction following absorption from the intestine it is probable that this fraction is concerned with the transport of copper to ceruloplasmin and also to various tissue copper proteins. Although the rate at which copper disappeared from the albumin component and appeared in the ceruloplasmin in both rat and man was partially dependent upon the dose administered, rapid uptake of copper in ceruloplasmin was apparent.

Summary. 1. The administration of Cu^{64} by mouth to the rat resulted in an immediate uptake of the copper by the albumin fraction of serum as indicated by experiments employing zone electrophoresis for the separation of the serum. Later the copper shifted to the major copper protein in rat serum, which has the approximate mobility of an α_1 globulin. 2. The administration of Cu^{64} by mouth to normal subjects and patients with cirrhosis of the liver also resulted in an immediate uptake of copper by the serum albumin fraction. The radioactivity shifted within a few hours to the α_2 globulin, ceruloplasmin, the major copper protein in human serum. 3. When radioactive copper was administered to patients with Wilson's disease, the radioactivity was confined to the albumin peak and little or no localization of the copper to the α_2 globulin fraction occurred.

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1. Laurell, C.-B., *Acta Physiol. Scand.*, 1947, v14, Suppl. 46.

2. Surgenor, D. M., Koechlin, B. A., and Strong, L. E., *J. Clin. Invest.*, 1949, v28, 73.

3. Cohn, E. J., *Experimentia,* 1947, v3, 125.

4. Holmberg, C. G., and Laurell, C.-B., *Acta Chem. Scand.*, 1947, v1, 944.

5. ———, *Scand. J. Clin. and Lab. Invest.*, 1951, v3, 103.

6. ———, *Acta Chem. Scand.*, 1951, v5, 921.

7. Gubler, C. J., Lahey, M. E., Cartwright, G. E., and Wintrobe, M. M., *J. Clin. Invest.*, 1953, v32, 405.

8. Bearn, A. G., and Kunkel, H. G., *ibid.*, 1952, v31, 616.

9. Scheinberg, I. H., and Gitlin, D., *Science,* 1952, v116, 484.

10. Denny-Brown, D., and Porter, H., *New England J. Med.*, 1951, v245, 917.

11. Bearn, A. G., *Am. J. Med.*, 1953, v15, 442.

12. Comar, C. L., Davis, G. K., and Singer, L., *J. Biol. Chem.*, 1948, v174, 905.

13. Kunkel, H. G., and Slater, R. J., PROC. SOC.

EXP. BIOL. AND MED., 1952, v31, 677.

14. Kunkel, H. G., and Tiselius, A., *J. Gen. Physiol.*, 1951, v35, 89.

15. Bearn, A. G., and Kunkel, H. G., *J. Clin. Invest.*, March 1954, in press.

16. Klotz, I. M., and Curme, H. S., *J. Am. Chem. Soc.*, 1948, v70, 939.

17. Earl, C. J., and Selverstone, B., personal communication to the authors.

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Autoradiographic Studies on Iron-59 Turnover by Erythroid Cells in Rat Bone Marrow. (20781)

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From the data on hemoglobin formation in the human bone marrow obtained by Thorell (1) with the aid of absorption microspectroscopy, it is known that "the endocellular synthesis of hemoglobin does not start before the ribose polynucleotide metabolism is finished" and that "the formation of the Fe-porphyrin component and probably also the simultaneous coupling to hemoglobin takes place chiefly during a later maturation phase." Since, from the data of Thorell(1), the ribose polynucleotide metabolism appears to be concluded within the basophilic phase of erythropoiesis, the polychromatic and orthochromatic erythroblasts appear to be the cells in which the Fe-porphyrin and hemoglobin appear first. He has evaluated the hemoglobin content in polychromatic erythroblasts to be 25%, in the orthochromatic 20-25%, and in the erythrocyte 33%, while the basophilic erythroblast either does not contain hemoglobin or the content is not measurable (less than 0.5%). These data, which agree with what is known about hemoglobin formation through the morphology and the staining of immature red cells, do not seem to be substantiated by the isotopic technic, since the rapidity with which the radioiron is taken up by the erythroid cell is so great that it is probable that the earliest stages of the erythroblasts (pro-erythroblasts and basophilic erythroblasts) are also actively involved in the formation of hemoglobin in the red cells. Clinical studies on patients with refractory anemias, showing uptake of iron in the bone marrow, but with little appearing in the peripheral red cells, also suggest that Fe must enter into the immature red cell precursors. This hypothesis can be verified only by using the qualitative procedure of autoradiography of bone marrow smears after radioiron administration. Other technics now available cannot discriminate between the different stages of erythropoiesis. There is no record of such an investigation with iron, although London and coworkers(2), Altman *et al.*(3), and Boyd and associates(4) have employed this technic to demonstrate that glycine labeled with C^{14} or N^{15} is taken up *in vivo* and *in vitro* by the immature red cells of the bone marrow and is incorporated into hemoglobin.

In the present study the stripping film autoradiographic technic, which gives the proper resolution for this purpose, was adopted to detect the first appearance of labeled iron in the earlier stages of erythropoiesis in the rat.

Method. A single dose of 80 μc of Fe-59-labeled ferric chloride in citrated solution was given intraperitoneally to 5 young rats of the Long-Evans strain, weighing about 160-180 g. The animals were then placed under ether narcosis, and sufficient bone marrow to make very thin smears was obtained from the femur or the tibia at specified intervals (½, 1, 3, and 6 hours). In order to obtain hyperplastic and hyperactive bone marrow, two additional animals were bled of half their

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* On leave of absence from Medical Pathology Institute, University of Padua, Italy, during tenure of Fulbright Fellowship at University of California, Berkeley.

TABLE I. Percentage of Labeled and Nonlabeled Cells in Rat Bone Marrow 6 Hr a Intraperitoneal Administration of 80 Microcuries $Fe\text{-}59Cl_3$.

	Negative	Positive				
	(−)	+	++	+++	++++	Total
Pro-erythroblasts	25	61	11	2	1	75
Basophilic erythroblasts	25	33	32	8	2	75
Polychromatic erythroblasts	12	17	49	20	2	88
Orthochromatic erythroblasts	0	9	38	49	4	100
Red cells	70	21	8	1	0	30

blood volume by heart puncture 4 days before administering 100 μc and 25 μc, respectively. After drying the smears at room temperature, the samples were fixed in pure methanol for 3 to 5 hours. In a darkroom, the smear surface was then covered with 1 cm x 1½ cm stripping film (NTB2 Kodak autoradiographic stripping film) by lifting the slide from beneath the film, which was floating in distilled water (the film must remain in water until thoroughly wet and swollen.) After complete drying in the dark, the slides were stored in a black box for 14 days and then developed with Kodak D19 developer for 3 hours at 20°C. After gentle washing in distilled water or 1% acetic acid (to break the developing), fixing took place in 20% sodium thiosulfate and 5% sodium bisulfite. After careful washing, the preparation was allowed to dry and then examined under the microscope in order to follow the Ag/Br reduction by the radioactivity. The smears were then stained with Giemsa solution through the film for 7 to 8 minutes. Such staining yields a fairly well differentiated preparation; even if the colors of the elements are slightly different from usual, a skillful observer can easily recognize the main characteristics of the cells.

The following characteristics were the most useful in differentiation of the erythroid series: (a) *Pro-erythroblast*: Diameter 12 μ, aspect very like the stem cell, large nucleus with one or two large nucleoli surrounded by a thin superficial layer of nucleolus-associated chromatin, thin cytoplasm of light blue color. (b) *Basophilic erythroblast*: Diameter 10 μ, characteristic chromatin and cytoplasm colors; no nucleus. (c) *Polychromatic erythroblast*: Diameter 8 μ, thicker chromatin and thinner layer of cytoplasm in which it is very difficult to demonstrate the first appearance of hemoglobin; with this technic the cytoplasm is usually uniformly blue. (d) *Orthochromatic erythroblast*: Diameter 6 μ, pycnotic nucleus; with this technic the cytoplasm appears mostly blue-greenish; sometimes it is lightly pink or yellowish.

Results. Since the activity estimation based on grain counting is always subjective, especially when there is, as in our case, a high background, the results will be based on the qualitative impression of the preparations, using quantitative determinations only to differentiate between the active and nonactive cells.

At ½ and one hour after iron administration in the normal rat, there is no definite evidence of activity in the cells, at least after 14 days' exposure of the stripping film and with the radioactivity of the injected material (80 μc). After 3 to 6 hours, radioiron appears in significant amounts in all stages of erythropoiesis as shown by the accompanying tables. From the data presented in Table I, it appears that labeled iron is present in detectable amount as early as in the pro-erythroblastic stage of red cell formation. It is not known if this is connected with hemoglobin formation, but the increasing content of radioiron in the more mature cells (100% of orthochromatic erythroblasts appear active after 6 hours) provides excellent indirect evidence of this assumption. The fact that red cells do not appear to be as active as the immature cells can be attributed to the fact that their population has been diluted by circulating red cells and consequently the results are not comparable.

In the rats rendered anemic by bleeding, the marrow appeared hyperplastic and hyperactive. The qualitative evaluation of the activity of the marrow from these animals is presented in Fig. 1 and Table II. In this

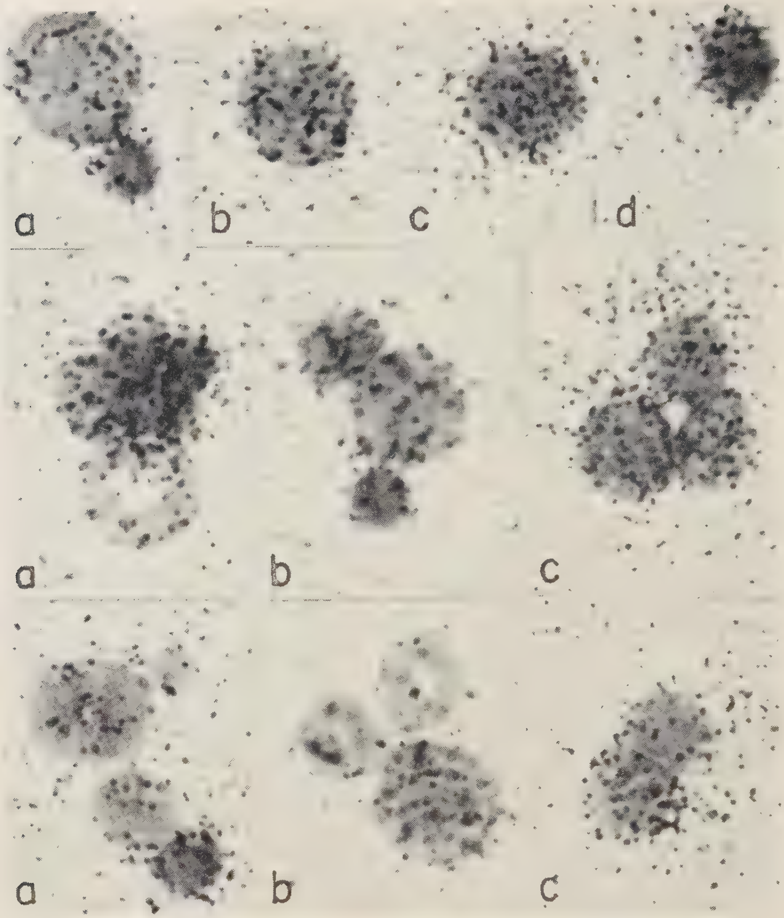

FIG. 1. Autoradiographic pictures of rat bone marrow 3 hr after intraper. inj. of 100 microcuries labeled Fe-59Cl$_3$ (stripping film autoradiography after 14 days exposure. Staining with Giemsa solution through stripping film, after development. Microphotographs at 1400 ×). In spite of poor differentiation of the element morphology and of the marked background (probably due to old stripping film or to lack of storage in refrigerator after exposure) the activity of labeled immature erythroid cells is evident even in earlier stages of erythropoiesis.

First row (from left to right): Erythroid elements with high activity. In (a) a pro-erythroblast and an orthochromatic erythroblast; (b) a basophilic erythroblast; (c) a polychromatic erythroblast; (d) orthochromatic erythroblast. 2nd row: Groups of erythroid cells with high activity. In (a) group of 4 polychromatic and orthochromatic erythroblast with almost complete darkening of film surface; (b) group of a pro-erythroblast, of a polychromatic and orthochromatic erythroblasts; (c) 3 basophilic elements with marked darkening. 3rd row: Groups of elements with lower activity. In (a) pro-erythroblast, a polychromatic and an orthochromatic erythroblasts with low activity; on the right upper corner an erythrocyte without a definite activity; (b) pro-erythroblast with moderate activity; (c) 2 polychromatic erythroblasts with fairly good activity.

TABLE II. Percentage of Labeled and Nonlabeled Cells in Anemic Rat 3 Hr after Intraper. Admin. of 100 Microcuries Labeled Fe-59Cl$_3$.

	Negative	Positive				
	(−)	+	++	+++	++++	Total
Pro-erythroblasts	63	32	5	0	0	37
Basophilic erythroblasts	18	55	24	3	0	82
Polychromatic erythroblasts	6	24	48	22	0	94
Orthochromatic erythroblasts	10	55	34	1	0	90
Red cells	93	7	0	0	0	7

case the peak is reached at the polychromatic stage (94%), and higher activity is revealed in the basophilic erythroblasts. These data should be considered in relation to relatively earlier sampling (3 hours), the higher radioiron dosage (100 μc), and the stimulation of bone marrow by previous bleeding.

The present investigation then shows that in the rat and with the doses used, there is a definite turnover of radioiron even by the earliest erythroid elements, namely pro-erythroblasts and basophilic erythroblasts. This was not known before, and it can be assumed as indirect evidence of the earliest stages of hemoglobin formation in the marrow.

Summary. The presence of iron-59 in the earliest stages of the erythroid series of the rat bone marrow was demonstrated by means of stripping film autoradiography after intraperitoneal injection of labeled ferric chlorides. Pro-erythroblasts and basophilic erythroblasts take up a detectable amount of radioiron after 3 to 6 hours. The maximum uptake, however, occurs in the polychromatic and orthochromatic stages.

The author is grateful to Dr. John H. Lawrence, Director, Donner Laboratory, for advice and assistance in research work and preparation of paper.

1. Thorell, B., *Studies on the formation of cellular substances during blood cell production,* H. Kimpton, London, 1947.

2. London, I. M., Shemin, D., and Rittenberg, D., *J. Biol. Chem.,* 1948, v173, 797.

3. Altman, K. J., Casarett, G. W., Masters, R. E., Noonan, T. R., and Salmon, K., *ibid.,* 1948, v176, 319.

4. Boyd, G. C., Casarett, G. W., Altman, K. J., Noonan, T. R., and Salomon, K., *Science,* 1948, v108, 529.

5. Elmlinger, P. J., Huff, R. L., Tobias, C. A., and Lawrence, J. H., *Acta Haematol.,* 1953, v9, 73.

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A Metabolic Study of Hydrocortisone in Rats. (20782)

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It has been demonstrated that slices of rat liver, kidney and spleen all effectively inactivate cortisone(1). Blood 17-hydroxycorticosteroid levels that have been elevated by administration of a single dose of cortisone or hydrocortisone to humans or experimental animals quickly decline to pretreatment values (2,3). Therefore, it seemed desirable to investigate the participation of the liver, kidney and spleen in the removal of exogenous 17-hydroxycorticosteroids from the blood of the rat *in vivo.*

Materials and methods. Male Sprague-Dawley rats weighing from 190 to 270 g were used without fasting. The removal of organs was carried out through a midline abdominal incision under light ether anesthesia. "Physiological hepatectomy" was performed by a modification of the method of Russell(4). The portal vein, hepatic artery and common bile duct were ligated after securing the gut from pylorus to descending colon in a mass ligature. This operation will be referred to henceforth as hepatectomy. Nephrectomy and splenectomy were accomplished after ligating and cutting the major blood vessels to these organs. The abdominal incision was closed with sutures. Control animals were subjected to laparotomy and a handling of the viscera. It was estimated that hepatectomy removed about 3 ml of blood from the circulation. To evaluate the effect of reduced blood volume upon 17-hydroxycorticosteroid levels, 3 ml of blood were withdrawn from the inferior vena cava in a group of rats through an abdominal incision, and absorbable gelatin (Gelfoam) was applied for hemostasis. Gelfoam was also placed in the peritoneal cavity of control animals for this group. Immediately following each operation, 0.5 ml of a hydrocortisone solution was injected into a tail vein. Solutions were prepared in concentrations of

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TABLE I. Plasma Levels of Hydrocortisone after Intravenous Injection of a Given Dose Following Hepatectomy, Nephrectomy, Splenectomy, or the Removal of 3 ml of Blood.

Operation	No. of rats	Avg body wt, g	Dose, mg	Time after inj., min.	Plasma hydrocortisone conc. (μg/10 ml)	S.E.	p
A. C*	6	212	.4	2	21	2	
H	7	223	"	"	34	3	$<.01$
N	6	216	"	"	16	2	$<.2$
S	6	226	"	"	17	2	$<.2$
B. C	7	206	"	10	4	1	
H	7	200	"	"	22	2	$<.001$
C	7	197	"	30	1	1	
H	7	195	"	"	26	2	$<.001$
C. C	7	191	"	"	3	1	
B	7	195	"	"	2	1	$>.5$
C	7	198	.0	"	0.7	0.2	
H	6	206	"	"	0.7	0.5	
D. C	6	269	1.6	10	24	1	
N	6	272	"	"	28	4	$>.5$
H	7	266	"	"	71	2	$<.001$
E. C	6	233	"	"	50	4	
S	7	242	"	"	48	3	$<.5$

* C = Control; H = Hepatectomy; N = Nephrectomy; S = Splenectomy; B = Blood removal.

0.8 or 3.2 mg per ml by dissolving a saline suspension of hydrocortisone[†] in 8 or 20% ethanol, respectively. A sample of aortic blood was drawn 2, 10 or 30 minutes later and analyzed for 17-hydroxycorticosteroids by the method of Nelson and Samuels(5).

Results. Controls. Considerable day to day variation was encountered in the plasma 17-hydroxycorticosteroid level of control animals injected with hydrocortisone under similar conditions. For this reason, the experiments in each section of Table I were all performed on the same day, using a single solution of hydrocortisone, and littermates for controls.

Preliminary studies in rats weighing an average of 230 g disclosed that, half a minute after the intravenous injection of 0.4 mg of hydrocortisone, the blood level rose from an average concentration of less than 1 μg per 10 ml to approximately 87 μg per 10 ml of plasma. At one minute after injection, the blood level had again fallen precipitously to about 37 μg per 10 ml of plasma, and thereafter declined at a much slower, fairly steady rate.

Hepatectomy. Two minutes after injection of 0.4 mg of hydrocortisone into hepatectomized animals the plasma level was found to be significantly higher than in the controls (Table IA). Ten and 30 minutes after injection the concentration fell to very low values in control animals, but remained high in hepatectomized animals (Table IB). In fact, there was no significant difference between the 10-minute and 30-minute determinations in the hepatectomized animals. The animals appeared to be weakened by hepatectomy, but were not in circulatory collapse. Simple removal of 3 ml of blood, approximating the amount that might be cut off from the circulation by the operation, was not found to interfere with the rapid fall in plasma hydrocortisone concentration following injection (Table IC). In the absence of injected hydrocortisone, there was no detectable accumulation of endogenous 17-hydroxycorticosteroids in hepatectomized rats within 30 minutes (Table IC).

Splenectomy and nephrectomy. Removal of the spleen or kidneys just prior to injection of 0.4 mg of hydrocortisone did not prevent the rapid fall in plasma concentration in 2 minutes (Table IA). Moreover, the concentration of hydrocortisone was not significantly different from that of control animals 10 minutes after the injection of 1.6 mg in nephrectomized (Table ID) or splenectomized (Table IE) animals; this larger dose was used in order to keep blood levels in a readily detected range at the longer time interval. Hepatectomized animals given the 1.6 mg dose for comparison showed much higher levels than the controls.

[†] Kindly supplied by Dr. Elmer Alpert of Merck and Co.

Discussion. Hepatectomized rats showed a fall in plasma hydrocortisone level within the first 10 minutes but not in the next 20 minutes. This suggests that the initial decline may have been produced by a distribution of the hormone throughout the body tissues, after which there was negligible removal of the compound from the circulation.

The stress of physiological hepatectomy could conceivably accelerate endogenous secretion of adrenal cortical steroids to a degree that this could in itself elevate blood levels. However, no significant rise in plasma 17-hydroxycorticosteroids was detected in hepatectomized animals that did not receive hydrocortisone.

Hepatectomy involved the simultaneous exclusion of the gastrointestinal tract and spleen as well from the circulation. However, splenectomy alone was without effect on the blood hydrocortisone level. The known high metabolic activity of the liver in degrading and excreting steroids(6,7) makes it highly likely that it, rather than the intestinal tract, plays the major role in producing the effects observed. The data do not distinguish between biliary excretion and transformations of hydrocortisone which would make it no longer detectable by the analytical procedure used.

The results with nephrectomized animals corroborate the findings of others(6,8) that very little exogenous 17-hydroxycorticosteroid is removed from the circulation by the kidneys.

Though the influence of the liver might be relatively less at physiological levels of hydrocortisone, the data presented indicate that this organ has a definitely greater capacity than other tissues in the metabolism of administered corticoid.

Summary. 1. Employing the analytical procedure of Nelson and Samuels for plasma 17-hydroxycorticosteroids, it has been shown that the intravenous injection of hydrocortisone in rats produces high concentrations of hormone, followed by a swift return to very low levels. 2. The rapid disappearance of exogenous hydrocortisone from the circulation is prevented by hepatectomy, but nephrectomy and splenectomy exert no appreciable effect.

The authors wish to acknowledge the technical assistance of Miss Germaine Dewhurst and Mrs. Kathleen Tendick.

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1. Louchart, J., and Jailer, J. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 393.

2. Nelson, D. H., Sandberg, A. A., Palmer, J. G., and Tyler, F. H., *J. Clin. Invest.*, 1952, v31, 843.

3. Samuels, L. T., and West, C. D., *Vitamins and Hormones,* New York, Academic Press, 1952, v10, 251.

4. Russell, J. A., *Am. J. Physiol.,* 1942, v136, 95.

5. Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metab.,* 1952, v12, 519.

6. Nelson, D. H., *Adrenal Cortex,* E. P. Ralli, Editor, Trans. Third Conf., New York; Josiah Macy, Jr. Foundation, 1952, p89.

7. Hechter, O., Solomon, M. M., and Caspi, E., *Endocrinology,* 1953, v53, 202.

8. Burstein, S., Savard, K., and Dorfman, R. I., *ibid.,* 1953, v53, 88.

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Effect of Diffusion Respiration on Duration of Vagal Cardiac Arrest. (20783)

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The always present danger of cardiac arrest during surgical procedures has stimulated a number of investigators within recent years to focus their attention to the etiological factors responsible for this catastrophe(1-4). The effects of anoxia, and more specifically, those of hypercapnia upon vagal cardiac inhibition have received particular attention as causative factors. Studies involving vagal stimulation during hypercapnia suggest that carbon dioxide retention during anesthesia may very greatly augment the effect of any vagal stimulation occurring during operative procedures. However, since previous studies of the effect of hypercapnia on cardiac vagal response have employed stimulation which could not be carefully quantitated, no relationship between the degree of hypercapnia and the duration of cardiac vagal asystole has been reported. The establishment of such an average relationship could well have value in predicting changes in cardiac response to vagal stimulation during respiratory acidosis. Such a correlation is reported in the succeeding paragraphs.

Methods. Since the hypercapnia occurring during a surgical operation is apt to have a gradual onset and is gradually progressive, it was felt desirable in these studies to avoid the precipitous changes in blood pH resulting from the abrupt exposure of animals to high concentrations of carbon dioxide. The method of diffusion respiration(5) was chosen as a means to produce a gradual, but progressive drop in arterial pH. Sixteen adult mongrel dogs of both sexes and varying weights were anesthetized with 35 mg per kg of body weight of sodium pentobarbital. Oxygen was administered endotracheally directly from a cylinder at 2 cm of water pressure through a system having 2 breathing bag reservoirs and a water valve pressure regulator. All of the animals were denitrogenated for one hour prior to the onset of diffusion respiration. During the latter half of the denitrogenation period, control determinations of the duration of vagal cardiac arrest and arterial pH were made. Diffusion respiration was induced in half of the animals by administration of a dose of sodium pentobarbital equal to the initial anesthetizing dose. Respiration was suspended in the second group by the intravenous injection of 0.35 mg per kg of body weight of decamethonium. Electrocardiographic recordings of the duration of vagal cardiac arrest and pH determinations were made at periodic intervals during the experimental period. Stimulation was applied to the right cervical vagus from a thyratron stimulator at 100 cycles per second and a voltage of such an intensity as to produce a minimum duration of cardiac arrest during the control period. This voltage was then used for the remainder of the experiment. The duration of cardiac arrest was determined by standard lead II electrocardiographic tracings. Blood was collected from the femoral artery and the whole blood pH determined by a Beckman glass electrode after allowing 3 minutes for equilibration with room temperature. Since the results are expressed as pH units change in blood reaction, no attempt was made to correct the readings to body temperature.

Results. Upon initiation of diffusion respiration with sodium pentobarbital, the duration vagal cardiac asystole decreased in every instance (Fig. 1). Following this initial decrease, the cardiac vagal response became progressively prolonged, with the response frequently becoming very pronounced at the lower pH levels. In all instances, the duration of cardiac vagal asystole was increased above the control values at the termination of the experiments. Although variability was observed between the behavior of individual animals, a decreased cardiac response to vagal stimulation immediately following the respiratory paralyzing dose of pentobarbital was observed in every experiment. The duration

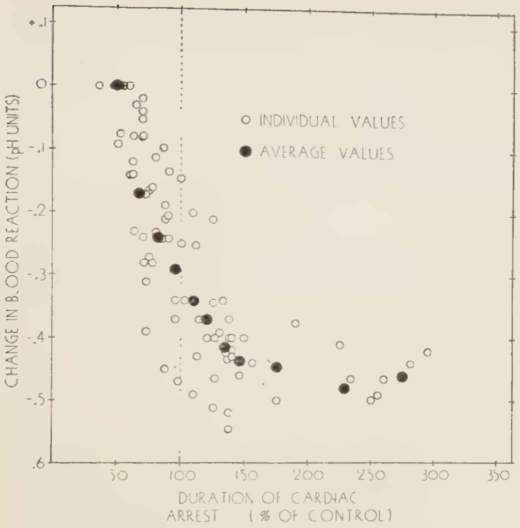

FIG. 1. Effect of respiratory acidosis, caused by diffusion respiration, on duration of vagal cardiac arrest. Diffusion respiration was induced in this series by an overdose of sodium pentobarbital.

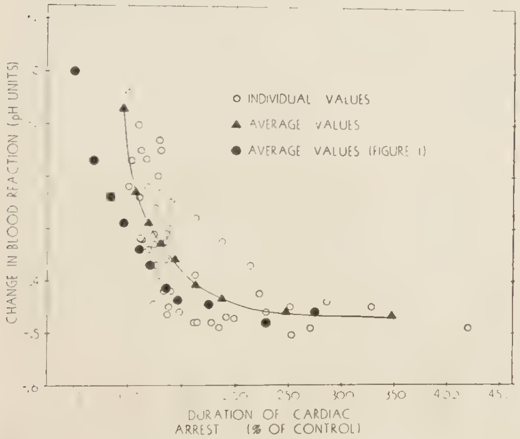

FIG. 2. Effect of respiratory acidosis, caused by diffusion respiration, on duration of vagal cardiac arrest. Diffusion respiration was induced by overdose of decamethonium. Average values from Fig. 1 are superimposed for purposes of comparison of results obtained with pentobarbital and with decamethonium.

of vagal cardiac asystole in this group usually did not return to the control level until the pH of arterial blood dropped in excess of 0.3 pH units.

Since this initially decreased response to vagal stimulation can probably be attributed to the anesthetic(6), a second group of animals was done using decamethonium—a compound which is known to have little effect upon the cardiac vagus(7). In this group, the duration of vagal asystole began increasing gradually from the beginning of the period of diffusion respiration, and became very pronounced at the lower pH levels achieved (Fig. 2).

Discussion. The duration of vagal cardiac arrest in these experiments never became markedly prolonged unless the arterial pH dropped in excess of 0.3 to 0.4 units. Although a paucity of information exists regarding the degree of acidosis which may occur during open chest surgical procedures, Young(4) has reported one case in which the arterial pH of a patient with a tetrology of Fallot dropped 0.3 pH unit during operation. If this could be regarded as an extreme instance(1), and if the above data could be regarded as applicable to the human, it would appear that a pH drop of that magnitude would not have an effect of great importance on vagal cardiac arrest. Such a conclusion seems to be substantially in agreement with investigations of vagovagal reflexes(3) which indicate that only transient cardiac slowing results from stimulation of intact vagal afferent fibers during severe hypercapnia. However, if greater decreases in arterial pH occur, especially in the presence of preexisting cardiac pathology or inadquate oxygenation, vagal mechanisms could well assume a role of some importance in initiating cardiac arrest during thoracic surgery.

Conclusions. 1. Sodium pentobarbital decreases the effect of vagal stimulation on the heart. Hence it is of considerable importance in experiments of this nature to maintain a constant level of barbiturate anesthesia in any one animal. 2. Progressive hypercapnia produced by diffusion respiration causes the duration of vagal cardiac arrest to increase progressively. This increase is very modest with decreases in arterial pH of the magnitude which could be expected in surgical procedures, but becomes especially marked at low pH levels. 3. The probable role of hypercapnia in surgical cardiac arrest is discussed.

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1. Beecher, H. K., and Murphy, A. J., *J. Thorac. Surg.*, 1950, v19, 50.

2. Harris, A. S., and Matlock, W. P., *Am. J.*

Physiol., 1947, v150, 493.

3. Sloan, H. E., *Surg. Gyn. Obst.*, 1950, v91, 257.
4. Young, W. G., Jr., Sealy, W. C., Harris, J., and Botwin, A., *ibid.*, 1951, v93, 51.
5. Draper, W. B., and Whitehead, R. W., *Anesthesia, and Analgesia,* 1949, v28, 307.
6. Shafer, G. D., Underwood, F. J., Gaynor, E. P., *Am. J. Physiol.*, 1930, v91, 461.
7. Paton, W. D. M., *Ann. N. Y. Acad. Sci.*, 1952, v54, 347.

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Acquisition of Topical Resistance to Thermal Injury.* (20784)

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Decrease in non-specific inflammatory reaction of tissues repeatedly stimulated by non-living irritating material, has been demonstrated(1-5). In a previous paper(4), we reported that an inflammation, produced by the injection of various irritants into the paw of a rat, protected this paw against a subsequent inflammation. When, after the first local inflammation had subsided, a second generalized hyperergic reaction was produced by an intraperitoneal injection of dextran or egg-white, the characteristic inflammatory edema appeared in the snout, ears and paws, but not in the pre-irritated paw. This permitted us to exclude the direct involvement of higher systemic regulations and to demonstrate this type of protection as being strictly topical.

In the investigation reported here, an attempt was made to obtain some data on the actual state of non-specific "sensitivity" or "reactivity" of pre-irritated tissues to a new inflammatory stimulus. Heat was used for the second irritation and was always given under compression of the blood vessels, thus making the tissues "poikilothermal" and ensuring an absolute uniform irritation in normal and pre-irritated tissues. This procedure eliminated the possibility, existing after a repeated administration of a chemical irritant, that through changes in tissue structures, the irritant might be mechanically prevented from acting uniformly upon the tissues. In this study, the influence of a preceding inflammation on the reaction to "poikilothermal heating" was investigated. The preceding inflammation was induced by the injection of edema-producing irritants, such as dextran or egg-white, into the rat paw, or by rubbing croton oil into the skin of the rabbit ear. Further, the influence of a pre-irritation by poikilothermal heating on the effect of a second, more intense heating was studied. Finally, the problem was investigated as to whether a pre-irritation by heat gives protection also against the reaction to a subsequent irritation by edema-producing chemical irritants.

Methods. Animals: Male Sprague-Dawley rats, weighing between 100 and 120 g, and male albino rabbits of 1.5 to 2.0 kg, were used. *Irritants*. Dextran was given in a saline dilution 1:37.5 of the commercial 6% solution (Macrodex). For local irritation, 0.2 ml was injected beneath the plantar aponeurosis of the hind paw of a rat; generalized hyperergic reaction was produced by the intraperitoneal injection of 1.0 ml. Egg-white was given in a saline dilution 1:5, 0.2 ml for local irritation, 1.0 ml intraperitoneally for generalized reaction. Croton oil, diluted 1:5 with oil (Mazola), was rubbed into the skin of the rabbit ear (2 drops on the upper and 2 drops on the lower surface) using rubber gloves. *"Poikilothermal heating." In rats.* Under compression of the aorta abdominalis with a rubber tube, the right hind paw was placed into 48°C water for 2 minutes, 15 seconds; the compression was then immediately released. For the subsequent irritation (the

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† Fellow of the Canadian Life Insurance Officers Association.

TABLE I. Topical Protection against Hyperergic Reaction to Dextran or Egg-White by a Preceding Inflammation in the Rat Paw; No Protection against Poikilothermal Heating. (6 rats in each group.)

	Paw	1st irritation (subcut.)	2nd irritation	Inflammatory index	% degree of protection in right paw
I	R*	.2 ml dextran	Heating both paws	48.5 ± 10.4	No protection
	L*	—	(48°C, 1′30″)†	37.8 ± 5.9	
II	R	*idem*	(*idem* for 2′15″)†	148.0 ± 18.7	„ „
	L	—		143.0 ± 16.8	
III	R	*idem*	Egg-white, 1 ml intraper.†	5.5 ± 5.0	97.8 ± 2
	L	—		235.2 ± 28.7	
IV	R	.2 ml egg-white	Heating both paws (49°C,	306.5 ± 20.6	1.0 ± 1.7
	L	—	2 min.) 24 hr later	310.5 ± 23.0	
V	R	*idem*	*idem* 48 hr later	312.8 ± 17.2	4.8 ± 3
	L	—		336.3 ± 16.8	
VI	R	*idem*	Dextran, 1 ml intraper.	5.8 ± 2.5	95.2 ± 3
	L	—	33 hr later	137.0 ± 31.2	

* R = Right; L = Left.
† 36 hr later.

time interval between the two irritations is individually indicated in the tables and legends), both hind paws, again under compression of the aorta, were placed into 49°C water for 2 minutes and the compression released immediately after. In order to minimize the differences in the duration of maximal tissue temperature which might occur if the volumes of the hind paws differed, the subsequent heating was given for 4 minutes at 47°C, if the right hind paw was still showing an increase in diameter due to the previous exposure to heat. By measuring the increase in the dorso-plantar diameter of the hind paws, at the level of the metatarsus, several times, before, during, and after the maximum reaction, an arbitrary inflammatory index (Σ% increase in diameter of all measurements) was determined for each paw. The comparison of the indices in the hind paws of the same animal permitted the assessment of the degree of protection as a percentage. *In rabbits.* The volume of both ears was measured, after shaving, by the water displacement in a test tube. The right ear was then placed into 49°C water for 2 minutes under compression of the blood vessels at the base of the ear, with rubber-armed curved arterial clamps. Immediately afterwards, the compression was released. (As there are differences in the reactions to this treatment in rabbits of varying ages and races, and at different room temperatures, the duration of the first heating should be arranged so that the inflammation does not exceed a 50% increase in ear volume, and so that the volume is almost back to normal after 2 days.) For the subsequent heating, both ears were clamped and placed, simultaneously into 49°C water for 2 minutes, by holding the animal horizontally upside down. The compression was released immediately afterwards. Estimations of the reaction and the degree of protection were made on the basis of the volume measurements in analogy with the procedure used for the rat.

Results. Topical protection against inflammation. A local inflammatory edema was elicited by the injection of dextran or egg-white into the right hind paw of rats (Table I). After gross signs of inflammation had disappeared, a second generalized hyperergic reaction in all 4 paws was produced by intraperitoneal injection of egg-white or dextran (Groups III and VI). In the right paw, previously subjected to an egg-white or dextran inflammation, the inflammatory edema did not occur. The preceding inflammation, however, gave no protection against a subsequent inflammatory reaction to heating, even if the heat irritation was very mild (Groups I and II), or made at different time intervals after the first inflammation (Groups IV and V). Also, an inflammation produced in the rabbit

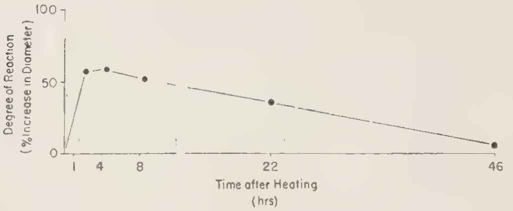

FIG. 1. Inflammatory reaction in hind paw of rats submitted to a poikilothermal heating, such as used for the pre-irritation in the following series of experiments (48°C, 2 min. 15 sec.). (Group of 6 rats.)

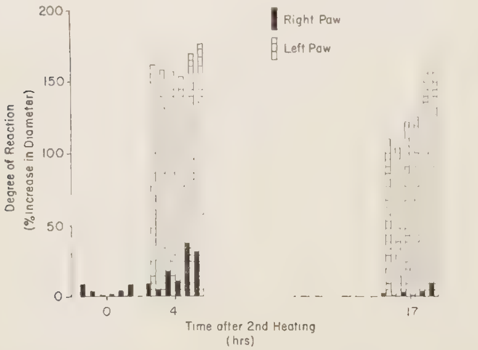

FIG. 2. Topical protection against heat injury by pre-irritation with heat. Inflammatory reaction in both hind paws of rats, submitted to poikilothermal heating (49°C, 2 min.). Right hind paw pre-heated (48°C, 2 min. 15 sec.), 2 days in advance. (Group of 6 rats.)

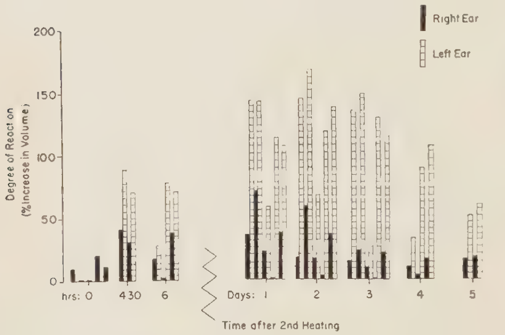

FIG. 3. Topical protection against inflammatory reaction to poikilothermal heating (49°C, 4 min.), in the rabbit ear. Right ear pre-heated (49°C, 2 min.), 3 to 5 days in advance. (Group of 5 rabbits.)

ear (5 rabbits) by rubbing it with croton oil, thus leading to a considerable swelling and to some superficial epithelial lesions, did not reduce the degree of a subsequent inflammatory reaction to heating (49°C, 4 minutes, 3-12 days later), given after healing of the epithelial defects and after disappearance of macroscopical signs of inflammation. The inflammatory index in the right pre-irritated ear was 634.0 ± 36.0, and 543.2 ± 50.0 in the left, non-pretreated ear. However, when tissues were pre-irritated by a mild heating, a marked topical resistance against a new and more severe heat injury was observed. The average degree of the inflammatory reaction following such a pre-irritation of a rat paw, is shown in Fig. 1. Sometimes, after pre-irritation, tissues showed only minimal signs of inflammation, even though a topical resistance against a new heat injury developed.

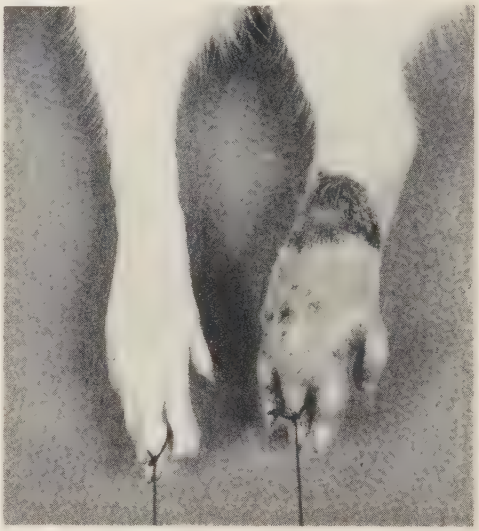

FIG. 4. Topical protection against necrosis. Poikilothermal heating of both hind paws (49°C, 2 min. 30 sec.). Right hind paw pre-heated (48°C, 2 min. 15 sec.), 3 days in advance.

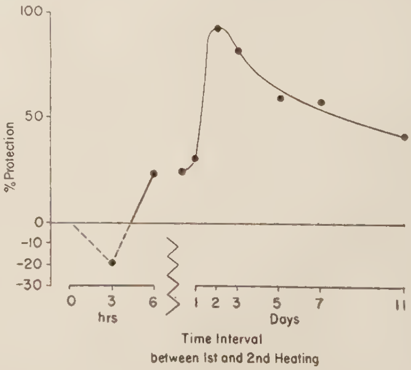

FIG. 5. Duration of the topical protection against inflammatory reaction to heat (47°C, 4 min. in the groups heated 3 hr, 6 hr and 1 day after pre-irritation, 49°C, 2 min. for the rest). Pre-irritation: 48°C, 2 min. 15 sec. (Groups of 6 rats each.)

TABLE II. Topical Protection against Hyperergic Reaction to Dextran and Egg-White by Poikilothermal Pre-Irritation. (Groups of 6 rats each.)

	Paw	1st irritation	2nd irritation (3 days later)	Inflammatory index	% degree of protection in right paw
I	R*	48°C, 2′15″	Dextran, 1 ml intraper.	5.0 ± 2.1	96.6 ± 1.4
	L*	—		132.8 ± 27.0	
II	R	*idem*	Egg-white, 1 ml intraper.	12.0 ± 8.9	93.3 ± 2.6
	L	—		157.5 ± 31.6	
III	R	*idem*	Dextran, .2 ml subcut.	265.0 ± 19.5	21.3 ± 3.2
	L	—	*idem*	346.0 ± 18.2	

* R = Right; L = Left.

The degree of this protection in the rat paw is shown in Fig. 2. Under these experimental conditions, the reaction in both paws was at the maximum approximately 4 hours after the second heating. Further, if a rabbit ear is pre-irritated by poikilothermal heating, it becomes, after macroscopical normalization, much less reactive to a new heat exposure (Fig. 3).

Protection against necrosis. If, by extending the duration of heating, the degree of tissue irritation is increased to a point where necrosis develops in the normal rat paw, the pre-irritated right paw will show but a mild inflammatory reaction and no necrosis (Fig. 4). Protection against the development of necrosis was observed also in the rabbit ear. Here, too, the right ear, pre-irritated by heating, showed only a mild reaction, whereas the normal left ear became necrotic if, in the second step, the duration of heating was extended or when an increased room temperature sometimes led to a stronger reaction to the second heating.

Duration of protection. The duration of the topical resistance after thermal pre-irritation against a new heat injury in the rat paw, is shown in Fig. 5. The protection appears to develop rapidly. After reaching a maximum, 2 days after the first irritation, the protection decreases in the form of a logarithmic curve.

Non-specificity of protection. The inflammatory edema formation, after intraperitoneal injection of dextran or egg-white in rats (Table II), is clearly inhibited in the paw which has been previously given a poikilothermal heating (Groups I and II). This may be due chiefly to a mechanical barrier of new structural elements which remained in the tissues after the first inflammation, and which, however, were almost ineffective against the concentrated action of locally injected dextran (Group III). Dextran, injected locally, produced an almost equal degree of inflammation in normal and in pre-heated paws. The ability of a pre-heated paw to react with acute edema formation can therefore be considered as being practically unchanged.

Discussion. There seems to be little doubt that heating under compression of the blood vessels brings both normal and pre-irritated tissues to the same temperature, over the same length of time. However, the tissues pre-irritated by heat show considerably less or almost no reaction, in comparison to normal non pre-irritated tissues, if an equal poikilothermal heating is given to both at the same time. Hence, it can be assumed that a mild pre-irritation by heat has produced some basic functional changes in the tissues thus making them indifferent towards the action of heat, which leads to inflammation and necrosis in normal tissues. Neither pre-irritation of tissues by the injection of irritants such as dextran or egg-white, leading to an acute edematous inflammation, nor a pre-treatment with croton oil, leading to a more chronic inflammation, provide protection against a subsequent poikilothermal heating and apparently do not produce such functional changes as ensue after pre-irritation by heat. This topically acquired indifference of tissues towards the action of heat, is probably a biological phenomenon similar to that of the adaptation to higher temperatures of lower forms of life, *e.g.*, Paramoecium and flagellates (6-8), by gradual exposure.

The tissue histamine content, even though decreased after poikilothermal pre-irritation in the skin of the rat paw, does not seem to be directly responsible for the protection against a new heating, since a depletion of the histamine in the rat paw by locally injecting compound 48/80 according to Feldberg and Talesnik(9), did not result in any protection against poikilothermal heating(10).

Protection against fatal shock in mice due to tourniquet of the hind legs has been reported when the same legs were submitted to a non-fatal tourniquet, 21 to 38 days in advance(11). In dogs and calves, subjected to burns, a rise in aminopeptidase activity was found in the lymph draining the burned area (12). Further, during the experiments reported here, it was found that the formation of hemolymphnodes in the popliteal region, generally occurring half an hour after poikilothermal heating of the rat paw and thus preceding the inflammatory swelling, was less or did not occur at all after the heating of the protected, pre-heated paw.‡ India ink particles, however, after poikilothermal heating, moved faster in the lymph vessels of the protected leg than they did in the normal side(13).

Therefore, it may be concluded that an irritation by heat leads to the exhaustion or blocking of a mechanism by which cytolytic substances are liberated from the injured tissues, or leads to the blocking of such substances. Thus, the reported data seem to give new emphasis to the findings of Beloff and Peters(14), who observed that a proteolytic enzyme, found in the skin of rats, was decreased after heating, presumably, according to the authors, because of an increased escape of the proteinase from the cells.

After local injection of egg-white or dextran into the rat paw, the inflammatory edema reaches its maximum within 20-30 minutes, while, after heating, this takes 3-4 hours, and thus the development of the inflammatory edema is here apparently dependent upon intermediary substances liberated in the tissues. Hence, the relatively unimpaired ability of pre-heated tissues to react with an acute edema formation as in the case of locally injected dextran, adds by exclusion further support to the assumption that the mechanism of protection against heat injury is mainly on the level of substances being dislocated and/or liberated normally after heat injury and inducing inflammation and necrosis.

Summary. 1. A topically acquired indifference of tissue cells towards the action of heat, such as leads to inflammation and necrosis in normal tissues, is described. This indifference was produced by a mild local pre-irritation of tissues with heat (immersion of a rat paw in 48°C water for 2 minutes, 15 seconds, or of a rabbit ear in 49°C water for 2 minutes). Heating was always done under compression of the blood vessels. 2. Local pre-irritation obtained by the injection of dextran or egg-white into the rat paw, or by producing a more chronic inflammation with croton oil applied on the rabbit ear, was ineffective in protecting the tissues locally against a subsequent heat exposure given under compression of the blood vessels. 3. After local pre-irritation of the rat paw by heat, a topical protection was observed also against the inflammatory reaction to intraperitoneally injected dextran or egg-white and, to a slight degree, to dextran subcutaneously injected into the paw.

I wish to thank Professor F. C. MacIntosh for his advice and interest in this work.

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‡ Hemolymphnodes were never observed after injection of egg-white or dextran into the rat paw.

1. Samuel, S., *Virchows Arch. f. path. Anat.*, 1892, v127, 467.

2. Stein, R., *Arch. f Dermat. u. Syph.*, 1909, v97, 47.

3. Jadassohn, J., and Rothe, L., *Berliner klin. Wchnschr.*, 1914, Nr. 11, 519.

4. Hoene, R., Labbé, P., and Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 65.

5. Selye, H., *Exp. Med. and Surg.*, 1953, v11, 81.

6. Dallinger, W. H., *J. roy. Micros. Soc.*, 1887, v1, 185 (cit. after Cameron, G. R., *Pathology of the Cell*, Oliver a. Boyd, Edinburgh, London 1952).

7. Jollos, V., *Biol. Zbl.*, 1913, v33, 222.

8. ———, *Z. indukt. Abstamm. u. Vereb. Lehre*, 1914, v12, 14 (cit. after Cameron).

9. Feldberg, W., and Talesnik, J., *J. Physiol.*, 1953, v120, 550.

10. Rocha e Silva, M., and Hoene, R., unpublished

data.

11. Rosenthal, S. M., Tabor, H., and Lillie, R. D., *Am. J. Physiol.*, 1945, v143, 402.

12. Zamecnik, P. C., Stephenson, M. L., and Cope, O., *J. Biol. Chem.*, 1945, v158, 135.

13. Hoene, R., unpublished data.

14. Beloff, A., and Peters, R. A., *J. Physiol.*, 1945, v103, 461.

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Blood Levels and Urinary Excretion in Normal Subjects after Ingestion of Tetracycline Analogues.*† (20785)

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The antibacterial action of the 3 chemically closely related antibiotics—tetracycline, oxytetracycline (Terramycin), and chlortetracycline (Aureomycin)—was found to be very similar against most of the strains of common human pathogenic bacteria that were tested (1). The results of assays of blood and urine for antibiotic activity following oral administration of these 3 agents are presented in this paper.

Materials and methods. Tetracycline (Achromycin) and chlortetracycline (Aureomycin) were supplied by Dr. Stanton M. Hardy of the Lederle Laboratories, and oxytetracycline (Terramycin) was provided by Dr. Gladys L. Hobby of Chas. Pfizer & Co. Each of these antibiotics was furnished in both capsules and tablets containing 250 mg equivalent of the free base; all were in the form of the hydrochlorides, except the oxytetracycline tablets, which were of the free base. The subjects were male members of the hospital staff who had not received any antibiotics for several weeks prior to the beginning of the study. Assays for antibiotic activity were done on citrated plasma from venous blood and on "clean" voided specimens of urine; 2-fold dilutions were made in brain-heart infusion broth (Difco pH ± 7.4) and seeded with an equal volume of a 10^{-4} dilution of an overnight growth of *B. cereus* No. 5 (obtained from Dr. A. C. Dornbush of Lederle Laboratories). The tests with plasma were read for visible growth after incubation at 37°C for 18 hours (and in some instances they were examined again after 24 and 40 hours); assays of urine were observed at 8, 18, and 24 hours. The results were recorded as the maximum final dilution completely inhibiting growth, as indicated by absence of visible clouding of the medium. The minimum inhibiting concentration in the specimens was determined by comparison with freshly prepared standard solutions assayed simultaneously and read at 18 hours.

Results. Two separate experiments were carried out. In the first, 12 subjects were given 1.0 g of each of the 3 analogs in rotation 4 to 7 days apart, 2 receiving capsules and 2, tablets of one of the 3 tetracyclines on each of the 3 days. None of the participants knew which of the analogs were given until the entire experiment was completed. Plasma and urine were collected at specified intervals, and those obtained at each interval were assayed simultaneously so that samples from all the subjects who had taken each of the 3 antibiotics were included in each test. The levels of antibiotic in the plasmas are shown in Fig. 1. None of those obtained before the doses showed inhibition of the test organism. More than one-half of the subjects had no demonstrable level at one hour, but the levels increased to a maximum between 2 and 6 hours. Two subjects who received tablets of oxytetracycline base showed no antibiotic

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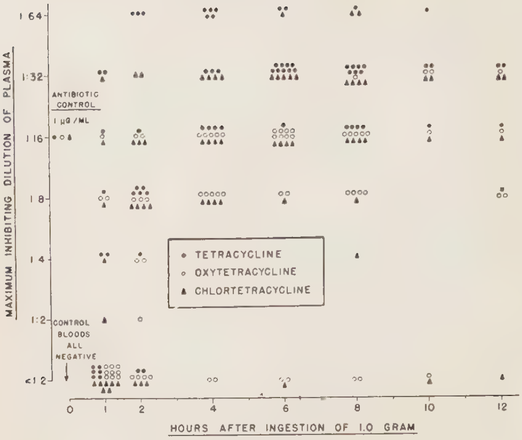
* Aided by a grant from the U. S. P. H. S.

† Summary of some of these findings presented at the Antibiotic Symposium, Washington, D. C., Oct. 28, 1953.



TEST ORGANISM = B CEREUS NO 5; INOCULUM = 10⁴/ML, INCUBATION 18 HOURS AT 37° C

FIG. 1.

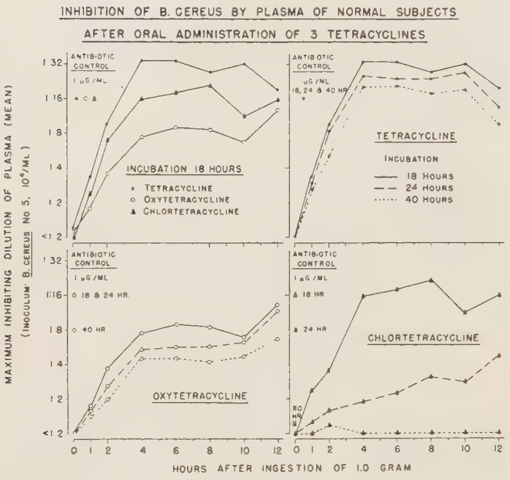


FIG. 2.

activity at any time. The levels for each of the antibiotics, as judged from the tests read at 18 hours, were quite similar, but they appeared to be highest after tetracycline and lowest after oxytetracycline. This is best shown in the average level as depicted in the upper left panel in Fig. 2. The effect of the rapid deterioration of chlortetracycline during incubation and the relatively greater stability of tetracycline in these tests are revealed in the other 3 panels in Fig. 2. The chlortetracycline was the least stable and deterioration proceeded rapidly after 18 hours, so that only minor inhibition of the test organism was demonstrated in the plasmas after 24 hours and none at all was discernible at the 40-hour reading. The least changes occurred in the plasmas from the subjects who received tetracycline and only slightly greater changes were noted in the successive readings of the tests in the plasmas of those who had taken oxytetracycline.

The results of the assays of urine obtained during the first 24 hours after the third dose are shown in Table I; these results are based on readings made after incubation for 18 hours. All of the antibiotics appeared rapidly in the urine, but the greatest variations in the concentrations and amounts excreted were noted in the first 2 hours. The maximum concentrations were obtained in most instances in the 2-6 hour specimens, but high concentrations were still found in the next 6 hours, and in some subjects even during the second 12 hours. The total amount recovered in the urine during the first 12 hours was lowest in the 2 subjects who took the oxytetracycline tablets, and varied in the others between 9% and 21% of the administered dose. During the second 12 hours, the amount excreted also varied in different subjects between 1.3 and 6.5% of the dose, but one of the subjects who took the oxytetracycline tablets excreted 24.3% of the dose during this period. The specimens of urine obtained just before the dose are not listed in the table, but they showed activity equivalent to 0.06-0.25 µg/ml in almost all of the subjects; this was the result of the continued excretion of antibiotic from the preceding dose, which was given 5 days previously. These amounts, however, when calculated for the daily output, could account for excretion of only a fraction of 1% of the administered dose. Individual specimens of urine obtained after the first 24 hours from one of the subjects who took tetracycline showed a concentration of 0.5 µg/ml on the 9th day, but none detectable on the 10th day; in a second subject, the equivalent of 0.5 µg/ml of tetracycline activity was found on the 5th day and none was detected on the 9th day. Oxytetracycline was found in urine obtained on the 5th day in a concentration of 0.25 µg/ml in one, and 2.0 µg/ml in another, and none was detected in specimens from either of these individuals on the 9th day.

TABLE I. Urinary Excretion of 3 Tetracyclines in Normal Adults after Ingestion of 1.0 g.

Subject	Antibiotic*	Conc., μg/ml of urine — Interval after dose (hr) 0–2	2–6	6–12	12–24	mg of antibiotic excreted — Interval after dose (hr) 0–2	2–6	6–12	12–24	% of dose excreted 12 hr	24 hr
C	T–c	40	320	160	—	2.4	53.8	29.2	—	8.5	—
D	”	40	320	320	40	3.2	64.4	76.8	12.6	14.4	15.7
J	T–t	320	320	320	160	17.6	10.6	67.2	64.8	9.5	16.0
K	”	320	640	320	160	35.2	96.0	81.6	63.2	21.3	27.6
E	O–c	80	320	160	80	4.8	49.6	41.9	17.2	9.6	11.4
F	”	320	320	160	—	10.2	41.6	37.6	—	8.9	—
L	O–t	20	80	320	1280	1.1	9.2	49.6	243.2	6.0	30.3
M	”	20	80	80	80	1.1	11.6	15.6	32.8	2.8	6.1
A	C–c	160	320	40	—	22.4	55.0	20.0	—	9.7	—
B	”	10	160	640	—	0.9	40.0	67.2	—	10.8	—
G	C–t	160	320	160	80	16.0	52.8	62.4	42.0	13.1	17.3
H	”	160	320	320	320	36.8	89.6	48.0	83.2	18.4	25.8

* T = tetracycline; O = oxytetracycline; C = chlortetracycline; c = capsules; t = tablets. All were hydrochlorides except oxytetracycline tablet which was the free base.

In the second experiment, done several weeks later, only capsules of the hydrochlorides were given. Only 11 subjects were available, 3 receiving chlortetracycline and the other 2 analogs each being given to 4 subjects. Five of these subjects had participated in the earlier experiment, but none had taken any antibiotics in the preceding weeks. Blood was obtained before the dose and at 1, 2, 4, 8, 12, 16, and 24 hours. A control specimen of urine was obtained before the dose and complete collections were made throughout the first 48 hours; single specimens were then obtained daily until no antibiotic could be detected by the test read at 18 hours (*i.e.*, until there was less than 0.06 μg/ml). The results based on tests read after incubation for 18 hours are shown in Fig. 3 and Table II. In this experiment, the levels obtained with tetracycline and oxytetracycline appeared to be similar, although higher levels were more frequent and more sustained with the former. Plasma levels were slowest to rise and earliest and most rapid to fall in the subjects who took the chlortetracycline; the earlier and more rapid deterioration of this antibiotic in these tests as compared with the observations made in the first experiment, may have contributed to this result. Significant concentrations of antibiotic were sustained in the blood even as long as 24 hours after the dose of either tetracycline or oxytetracycline.

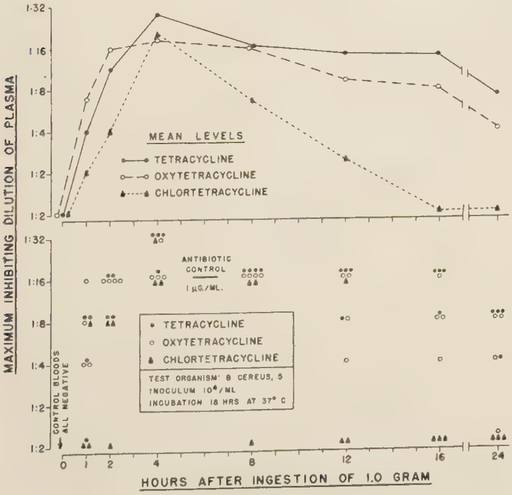


FIG. 3.

The urinary excretion of the 3 analogs during the first 24 hours, as shown in Table II, was similar to that observed in the first experiment. Similar variations were noted among subjects who received each of the 3 antibiotics, both with respect to the concentrations and the amounts excreted at different intervals. In this experiment, no inhibitory activity on the test organism was detected in the urine of any of the subjects just before taking the dose. The concentrations of antibiotic and the amounts excreted appeared to be lower after the first 24 hours in the subjects who had taken chlortetracycline. Excretion of the antibiotics continued in almost all the subjects through the 8th day; in 2 who took tetracycline, and in one who took oxytetracycline, some activity was still detected in urine obtained on the 10th day.

TABLE II. Urinary Excretion of 3 Tetracyclines after 1.0 g Orally Given as Capsules of the Hydrochlorides.

Subject	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Antibiotic	Tetracycline				Oxytetracycline				Chlortetracycline		
					Conc. in urine, μg/ml						
Before dose	0*	0	0	0	0	0	0	0	0	0	0
0- 2 hr	80	80	160	20	320	320	320	320	10	320	80
2- 4	320	640	160	160	320	160	320	640	80	320	320
4- 8	320	320	320	160	320	80	320	640	160	160	640
8-12	320	320	160	160	160	80	640	320	40	160	160
12-16	160	160	80	80	80	40	320	320	20	40	160
16-24	80	160	80	80	80	40	160	160	40	80	80
24-36	40	40	40	20	40	20	80	80	5	10	20
36-48	20	20	20	10	20	10	40	20	1	5	10
3 day	8.0	4.0	—	—	4.0	2.0	4.0	8.0	.5	.5	2.0
4	4.0	4.0	2.0	2.0	2.0	2.0	2.0	1.0	.06	.06	2.0
5	1.0	2.0	1.0	1.0	1.0	1.0	1.0	.5	.06	.13	.25
6	.5	.5	.13	.13	.25	.5	—	.25	0	.13	.13
7	.25	.25	.25	.25	.25	.25	.25	.13	0	0	.13
8	.13	.13	.13	.13	.13	0	.13	.13	0	.13	.06
9	.13	0	0	.13	.06	0	.13	.06	0	0	.06
10	.06			.13	0		.06	0			
11	0			.06	0		0	0			
					% of dose excreted						
12 hr	12.4	17.8	14.1	7.9	10.1	5.8	17.9	16.5	4.6	14.1	19.4
24	18.5	23.0	18.5	10.2	13.8	8.0	26.6	24.5	6.8	17.6	26.7
48	21.0	25.2	22.2	11.5	17.1	9.5	31.3	26.8	7.3	18.7	28.0

* <0.06 μg/ml.

TABLE III. Toxic Effects Observed in 12 Normal Adult Male Subjects after Ingestion of 1.0 g of Each of 3 Tetracyclines.

Subject	Antibiotic Tetracycline	 Oxytetracycline	 Chlortetracycline
		Capsules	
A	*1. None	2. None	3. None
B	1. "	2. Epigastric distress, began in 24 hr, lasted 3 days	3. Epigastric distress & nausea after 24 hr, lasted 48 hr
C	3. "	1. Diarrhea, 4 days	2. None
D	3. "	1. None	2. Bulky stools
E	2. "	3. "	1. None
F	2. 3-4 bulky stools	3. Flatulence	1. "
		Tablets	
G	1. None	2. Diarrhea	3. None
H	1. "	2. Diarrhea, tenesmus, lasted 3 days	3. Tenesmus, diarrhea next day
J	3. "	1. Nausea & diarrhea, 1 day	2. None
K	3. "	1. Flatulence for 36 hr, then diarrhea	2. Diarrhea, ? from previous dose, lasted 36 hr
L	2. "	3. None	1. Nausea, mild abdominal distress for 48 hr
M	2. "	3. "	1. Bulky stools, 24 hr

* Number represents the order of administration, 4 to 7 days apart.

Untoward effects. The symptoms experienced by the subjects and attributable to the antibiotics during the first experiment are listed in Table III. These findings confirm those observed in clinical studies which indicated that the major untoward effects of each of the tetracyclines are referable to the gastrointestinal tract and that, of these, diarrhea is the most serious; they also confirm the observations made in this hospital that such gastrointestinal effects are most frequent with oxytetracycline and least frequent with tetracycline(2). In the present experiment there were essentially no untoward effects from tetracycline, only one of the subjects having passed 3 or 4 bulky stools on the day after the dose. Diarrhea, flatulence or epigastric distress in various combinations were experienced by 7 of the subjects who took oxytetracycline and by 4 of those who took chlortetracycline, and 2 additional subjects passed some bulky stools after doses of the latter. In 3 of the subjects who manifested gastrointestinal symptoms from chlortetracycline including 2 with diarrhea, the chlortetracycline was given after oxytetracycline and similar symptoms were experienced with the previous dose.

In the second experiment one of the subjects who took oxytetracycline had 4 watery bowel movements on that day and 2 to 4 similar watery movements during each of the next 4 days; this individual had not participated in the earlier experiment. One subject experienced mild nausea immediately on taking the tetracycline capsules and another after taking the chlortetracycline, both on an empty stomach; in both subjects the nausea cleared promptly after they began to eat breakfast and no other symptoms were experienced by these or by any of the other subjects.

Discussion. In the previous paper(1) it was shown that the 3 tetracyclines have very similar activity against common pathogenic bacteria, but that they differed in their stability and, to some extent, also in their activity against individual strains or against certain species of bacteria. The relative instability of chlortetracycline under the conditions of the *in vitro* inhibition tests, or in assays of the antibiotic in body fluids, may be of paramount importance in making comparisons of the absorption and excretion of the 3 tetracyclines. In the data presented here, this is clearly brought out in comparing the blood levels. That the same phenomenon is not necessarily true, at least to the same extent, *in vivo,* is apparent from the fact that chlortetracycline continues to be excreted for several days after a single dose, and in concentrations not markedly dissimilar from those observed with the other 2 analogs which are more stable *in vitro.* It has also been shown that in embryonated eggs, chlortetracycline does not deteriorate nearly so rapidly as it does in culture media kept at the same temperature(3), and a substance has been found in egg yolk which apparently protects the chlortetracycline from deterioration(4). It seems not unlikely, therefore, that similar protection against deterioration is available in tissues and body fluids in which, despite their slightly alkaline reaction, chlortetracycline appears to persist in active form for several days. On the whole, however, blood levels from oral tetracycline appear to be somewhat higher and more sustained and its excretion appears to be slightly prolonged over that observed following similar oral doses of oxytetracycline and chlortetracycline.

Conclusions. Blood levels after ingestion of a single dose of 1 g of tetracycline are somewhat higher and better sustained than those resulting from a similar dose of either oxytetracycline (Terramycin) or chlortetracycline (Aureomycin). High concentrations appear rapidly in the urine after ingestion of each of these 3 analogs and persist over a period of 48 hours. After that, appreciable concentrations of these antibiotics continue to be excreted for more than a week after a single oral dose of 1.0 g. Untoward gastrointestinal symptoms, particularly diarrhea, were frequent after a single oral dose of 1.0 g of oxytetracycline, they were observed, but less frequently after chlortetracycline, and did not occur after the same dose of tetracycline.

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1. Love, B. D., Jr., Wright, S. S., Purcell, E. M., Mou, T. W., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 25.

2. Finland, M., *et al.*, *J.A.M.A.*, in press.

3. Womack, C. R., Kass, E. H., Wells, E. B., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 706.

4. Womack, C. R., Kass, E. H., and Finland, M., *J. Lab. and Clin. Med.*, 1950, v36, 655.

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Uptake of Ammonia by the Brain in Hepatic Coma.[*] (20786)

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(Introduced by R. W. Berliner.)
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The probable causal relation between the development of hepatic coma in terminal liver disease or in Eck fistula patients and the rise in blood ammonia(1) raises the question of whether the ammonia acts as a toxic agent by the inhibition of some enzyme system in brain, or whether it exerts its effect by combination with an integral cerebral metabolite.

A study of the cerebral arteriovenous difference of ammonia in a number of patients both comatose and non-comatose, with and without apparent liver disease, reveals a striking difference between patients with high blood ammonia levels and those with normal levels.

Methods. Blood ammonia is determined by a microdiffusion-Nesslerization method modified from Seligson(2) on freshly drawn blood from the femoral artery and the jugular bulb. The normal range for venous blood by this technic is from 0.30 to 1.00 γ/ml. In severe hepatic disease this level is elevated, although methods which yield higher normal values through the inclusion of ammonia formed during the determination itself tend to obscure this finding(3).

Results. Table I shows the ammonia levels in the arterial and jugular bulb blood of 3 non-comatose patients, 3 cases of liver failure, and 3 cases of coma or semi-coma without apparent liver disease. In the non-hepatic cases there is no significant uptake of ammonia, and in the cases with liver disease there is a marked uptake. This phenomenon is consistent throughout all cases studied, and all cases with elevated blood ammonia show an uptake. The arteriovenous difference is roughly proportional to the arterial value.

In view of this "utilization" of ammonia, the following hypothesis is suggested. The

TABLE I.
Arterial and Cerebral Venous Blood Levels of Ammonia (Gamma per ml of Whole Blood).

				NH_3 N., μg/ml		
Patient	Age	Diagnosis		Femoral artery	Jugular bulb	Diff.
		Awake				
M.W.	35	Old CVA	Alert	.92	.97	— .05
F.R.	53	Chronic asthmatic	”	.75	.73	.02
W.J.	77	Parkinsonism	Senile	.84	.82	.02
						.00
		Non-hepatic				
R.G.	23	Post anoxic	Coma	.60	.63	— .03
I.C.	88	Hypertensive enceph.	Semicomatose	.33	.31	.02
W.J.	73	Arteriolar nephrosclerosis	Comatose	.80	.88	— .08
						— .03
		Liver disease				
T.B.	51	Cirrhosis, Eck fistula	Semicomatose	8.44	7.12	+1.32
V.W.	65	Cirrhosis	Deep coma	2.81	2.50	+ .31
L.P.	59	”	Semicomatose	3.00	2.32	+ .68
						+ .77

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[*] Aided by grant from the Playtex Park Research Foundation.

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ammonia enters the brain and combines with alphaketoglutaric acid by the reversal of glutamic dehydrogenase. This abstracts large amounts of the keto acid from the Krebs cycle, preventing regeneration of oxalacetate and further oxidative metabolism. The marked diminution of cerebral oxygen uptake which we have seen in hepatic coma(4) can thus be explained on the basis of the failure of the major oxidative pathway in the brain.

Summary. The arteriovenous difference of ammonia has been investigated in a variety of conditions and has been found to be elevated in hepatic coma, as has been the arterial level of blood ammonia. The evidence for a significant uptake of ammonia in comatose patients with liver disease suggests a mechanism for hepatic coma. This mechanism could be the reductive amination of ketoglutaric acid to form glutamate with the concomitant removal of significant amounts of ketoglutarate, preventing Krebs cycle regeneration.

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1. Salaskin, Z. F., *Physiol. Chem.*, 1898, v25, 128, 449; McDermott, W. V., Jr., Adams, R. D., *J. Clin. Invest.*, 1954, v33, 1.

2. Seligson, D., and Seligson, H., *J. Lab. and Clin. Med.*, 1951, v38, 324.

3. Schwartz, R., Phillips, G. B., Gabuzda, G. J., Jr., and Davidson, C. S., *J. Lab. and Clin. Med.*, 1953, v42, 499.

4. Fazekas, J. F., and Bessman, A. N., Unpublished data.

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A Simple Method of Gradually Producing Permanent Occlusion of Portal Vein in the Dog.* (20787)

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In our studies on the effect of interference with the arterial and venous blood circulation to the liver we have found a simple method of gradually producing a permanent occlusion of the portal vein which could likewise be used to advantage in other similar problems.

Acute complete occlusion of the portal vein by any method is not tolerated by the dog and is regularly fatal. To produce portal occlusion, a procedure has to be used which leads to gradual closing of the vein. Wrapping of irritative cellophane around the vein is unsatisfactory, because it causes marked adhesions and usually only incomplete closure of the vein. Partial constriction of the portal vein with a ligature of some nonabsorbable material leads occasionally to complete obstruction of the vein but is frequently without effect.

We have found that placing two constricting ties of umbilical cord (cotton No. 070 Bauer and Black, Chicago), around the portal vein at a distance of 0.3 to 0.5 cm, invariably leads to complete and permanent occlusion of the portal vein within 1-2 weeks. The ligatures should be tied as tightly as possible over the tip of a small curved hemostat. After withdrawal of the hemostat, the bowels are not cyanotic, and the afferent portion of the portal vein is not appreciably distended.

As a rule, this procedure is well tolerated. In all experiments, reoperation showed complete occlusion of the portal vein by a clot localized at the ligatures.

Summary. Gradual complete occlusion of the portal vein in the dog is obtained by placing two constricting ties around the portal vein. The procedure is recommended when gradual and complete permanent occlusion of the vein is desired.

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Serial Propagation of *Toxoplasma gondii* in Roller Tube Cultures of Mouse and of Human Tissues.* (20788)

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The demonstrated usefulness of roller tube cultures of mammalian tissue for the isolation of certain viruses of human origin has stimulated interest in the behavior of other types of human pathogens in tissue culture. While multiplication of *Toxoplasma gondii* has been demonstrated in cultures of chicken embryonic tissues(1-6) and on a single occasion in mammalian tissue(7), the propagation of *T. gondii* in cultures of the roller tube type has not yet been explored. Apart from the diagnostic considerations involved, a study of the capacity of *T. gondii* to propagate in roller cultures of human and of other mammalian tissues might be expected to augment our knowledge of the bionomy and the pathogenic attributes of the parasite. This paper deals in a preliminary manner with the behavior of *Toxoplasma* in roller cultures of mouse embryonic and various human tissues.

Materials and methods. A. *Preparation and maintenance of cultures.* Roller tube cultures were prepared as described by Robbins, Weller and Enders(8) in studies on cultivation of the poliomyelitis viruses. The tissues employed were either of mouse embryonic origin, derived from white mice of the Harvard Medical School strain between the 13th and 18th day of gestation, or were of human origin, including foreskin, myometrium and embryonic skin and muscle. The mouse embryos or individual human tissues were minced with scissors and fragments explanted in a thin layer of clotted chicken plasma on the walls of roller tubes or on narrow coverslips which were subsequently inserted into roller tubes. The fluid medium of Enders(9) (beef amniotic fluid, 90%; beef embryo extract, 5%; and inactivated horse serum, 5%) with penicillin, streptomycin, soybean trypsin inhibitor and phenol red(8), was employed. All roller cultures were maintained at 35°C and the nutrient fluids (2 ml per tube) were changed at 3- to 4-day intervals. Cultures were inoculated as tissue growth became well established, usually after 4 to 8 days incubation in the case of the mouse or human embryo preparations, and after 10 days for the cultures of human prepuce and myometrium. For histologic examination coverslip preparations were withdrawn at intervals, fixed in Zenker's-acetic or Bouin's, and stained *in toto* with hematoxylin-eosin or Giemsa's stain. B. *Strains of Toxoplasma employed.* The RH strain was supplied by Dr. Quentin M. Geiman in whose laboratory it had been maintained by intraperitoneal passage in mice at 4- to 5-day intervals since its receipt in February, 1953, from Dr. Leon Jacobs of the National Institutes of Health. A second strain (KM), recently isolated at the Children's Hospital by Dr. William D. Winter, Jr. from a fatal case was supplied by him in the form of material from the 24th mouse passage. C. *Inoculation and subculture procedures.* The inoculum for the first passage in roller cultures consisted of 0.1 ml of a 1:20 dilution in medium of peritoneal fluid removed from an infected mouse on the third day of infection. Subsequently, as organisms became numerous in the medium, (determined by microscopic inspection), fluids from a group of infected tubes were pooled and 0.1 ml aliquots transferred to each of a new group of 3 or 4 roller cultures. Comparable control cultures were maintained and subinoculation of fluids from such uninfected cultures was performed at the time of serial passage. Routinely, aliquots of materials used for transfer were cultured on blood agar plates and in thioglycollate broth.

Experimental observations. A. *Propagation of the RH and KM strains of Toxoplasma in mouse embryo roller cultures.* The two strains behaved similarly *in vitro* and serial passage of each was accomplished without difficulty.

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* Aided by a grant from the National Foundation for Infantile Paralysis.

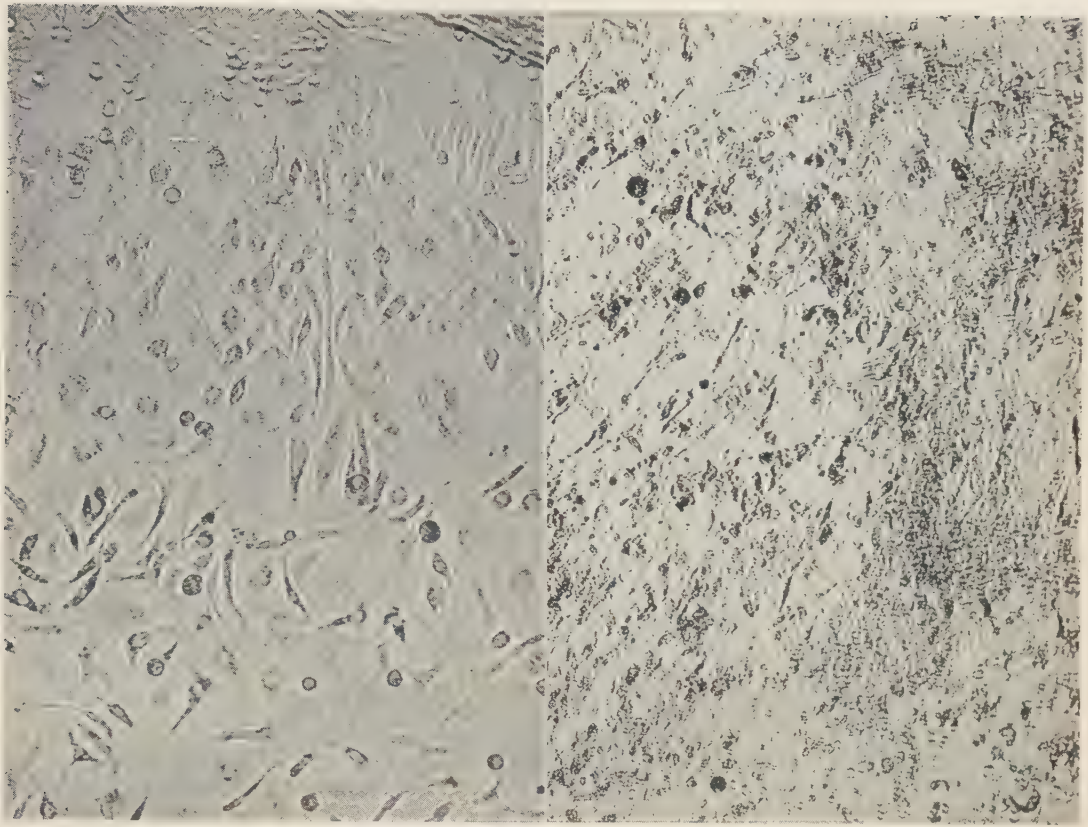

FIG. 1. Photographs (× 150) of 15-day-old mouse embryonic roller cultures. Left: control showing normal cellular growth. Right: extensive cellular destruction 11 days after inoculation with *Toxoplasma.*

The RH strain has been maintained *in vitro* for 15 consecutive passages during a cumulate period of 81 days while the KM strain has been propagated for 7 passages over a 56-day period. It is to be noted that the manipulations carried out during the maintenance of the RH strain for 15 passages resulted in a dilution of the order of 10^{-37} of the initiating inoculum; yet, the LD_{50} of infected fluids from the 15th passage as determined in mice was $10^{-5.5}$ per ml.

After inoculation cultures were usually examined daily employing a magnification of 100 X. Organisms floating free in the medium were easily recognizable and usually appeared in small numbers between the 3rd and 5th day after inoculation. Thereafter, the number of free organisms increased rapidly reaching maximum levels around the 6th or 8th day; at this time the organisms appeared as loose aggregates or floating plaques which sometimes extended across the breadth of the low-power microscopic field. Such cultures contained as many as 2.5 million organisms per ml of medium as ascertained by counts with a haemocytometer. It should be noted that infrequently, for reasons not now understood, organisms were first observed 10 to 12 days after inoculation.

Striking degenerative changes, readily visible under low magnification, occurred in the tissue phase of the cultures coincident with the appearance of large numbers of *Toxoplasma* in the medium. Typically, diffuse fragmentation and death of the cell population developed over the 48- to 96-hour period after free organisms were first observed. These specific cytopathic alterations in cultures of mouse tissue are illustrated in Fig. 1. At this stage a difference between the pH of the medi-

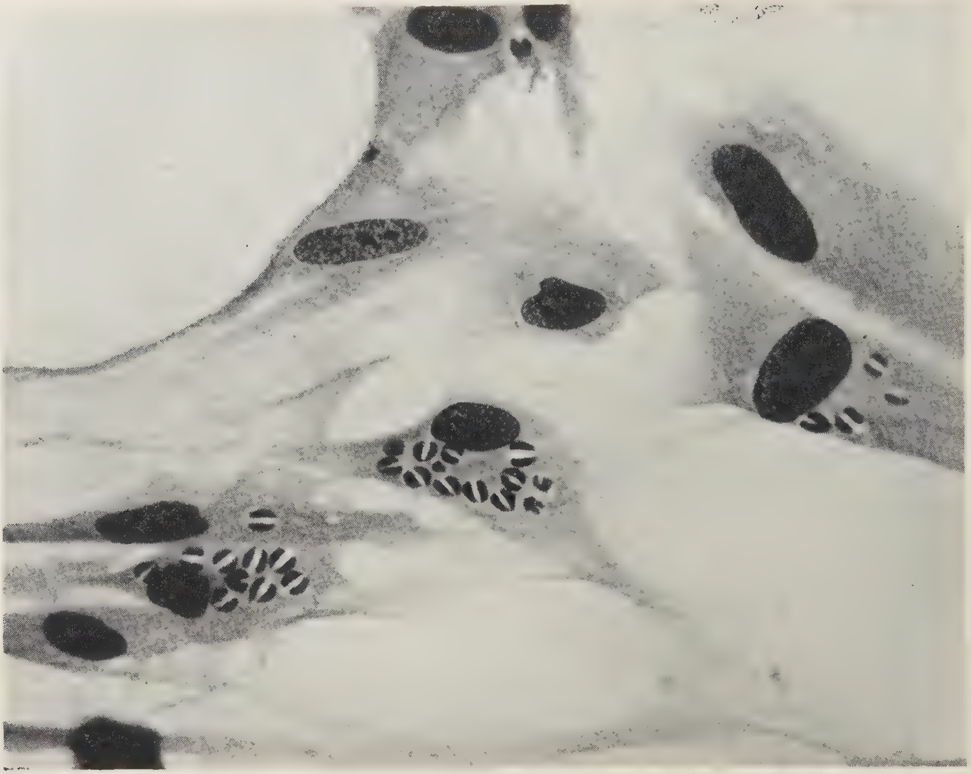

FIG. 2. *Toxoplasma* in roller culture of human uterus 4 days after inoculation. Coverslip preparation. Giemsa. × 600.

um in inoculated and in control cultures was also present, reflecting the marked destruction of tissue in infected tubes. Characteristically, the number of organisms in the medium declined rapidly once the tissues were destroyed.

In addition to the observations made during passage of the two strains, a series of ancillary experiments have been performed that will be described in detail elsewhere. Certain of the findings may be summarized as follows:

1) In confirmation of earlier reports, multiplication of *T. gondii in vitro* has not been observed in the absence of living cells. Negative results were obtained following inoculation of tubes containing medium alone, or of cultures in which the tissue phase had previously been killed with heat. 2) While study of stained preparations is not complete, all types of cells present in the mouse embryo cultures appear susceptible to the cytopathic effect of *T. gondii.* Evidence of renewed cell growth in cultures following generalized specific destruction was not noted during an extended period of observation. 3) No evidence has yet been educed indicating that a soluble toxic substance is responsible for the cytopathic changes. When the medium in uninfected cultures was replaced by medium from heavily infected cultures that had been rendered free of organisms by centrifugation, no obvious changes were noted in the metabolic activity or growth rate of the tissues. 4) No significant alteration of virulence of *T. gondii* for mice has been observed thus far in the course of serial cultivation *in vitro.* Tenfold dilutions of fluids from the 7th roller tube passage of the RH strain in mouse tissue were inoculated into roller tubes and also intraperitoneally in mice; the ID_{50} and LD_{50} titers respectively were of the order of 10^{-6} per ml. Two titrations performed on infected fluids from subsequent passages again indicated an LD_{50} titer for mice of the order of 10^{-6}; however, the ID_{50} titers in roller cultures of these materials were significantly lower.

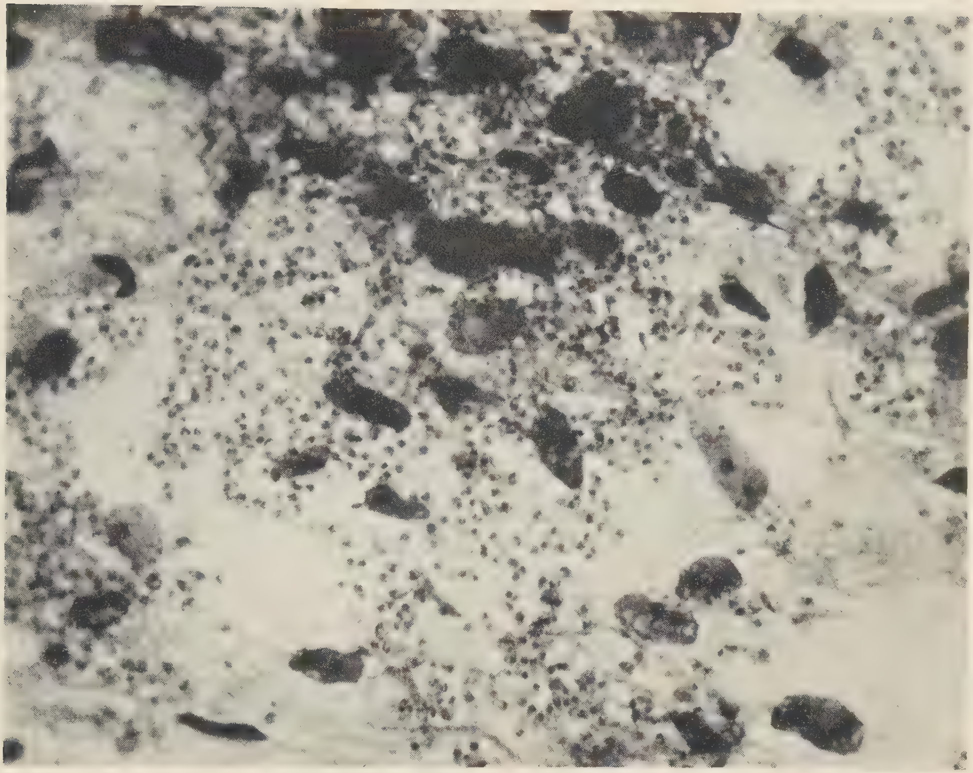

FIG. 3. Roller culture of human foreskin 7 days after inoculation with *Toxoplasma,* showing extensive tissue destruction and numerous extracellular organisms. Coverslip preparation. Giemsa. × 600.

B. *Growth of the RH strain in various human tissues.* This phase of the study was designed to determine if *T. gondii* derived from infected mouse embryo tissue cultures could multiply in various human tissues *in vitro.* Growth of *Toxoplasma* was readily demonstrated in roller tubes containing coverslip preparations of human uterus (Fig.2), foreskin, or embryonic skin-muscle. In each of these, as in mouse tissues, multiplication was associated with a generalized cytopathic effect (Fig. 3). Furthermore, the organism was propagated for 3 passages in roller cultures of human myometrium, during which time its pattern of multiplication and cytopathogenicity generally paralleled that in mouse embryo cultures.

Discussion. Multiplication of *T. gondii* has been conclusively demonstrated in roller tube cultures of mammalian cells. The findings serve indirectly to confirm Lock's(7) note on the growth of this organism in embryonic rat heart tissue. That *T. gondii* grows readily in cultures of various human tissues has been determined. Moreover, so far as we are aware, organisms of the RH strain have for the first time been grown in tissues similar in origin to those from which they were originally isolated some 15 years ago.

Although previous workers have noted degenerative changes in cultures infected with *Toxoplasma,* the relationship between multiplication of this organism *in vitro* and concomitant destruction of actively proliferating tissue has not been generally recognized. As in the case of certain viruses, the cytopathogenic attributes of *Toxoplasma* may be utilized as an indicator of specific biological activity in cultures. The large number of organisms released into the fluid phase of the cultures suggests that such material may provide a useful source of antigen for various immunologic procedures. In contrast to the organisms in infected mouse peritoneal fluid, many of which are intracellular, those in tissue culture fluid are almost completely extracellular.

The demonstrated ability of *Toxoplasma* to multiply *in vitro* in mammalian cells also suggests that this organism may be encountered on occasion by those utilizing tissue culture procedures for the isolation of viruses from man. In such cultures, the phenomena associated with the multiplication of *Toxoplasma* might be erroneously ascribed to bacterial contamination or to viral cytopathogenicity.

Summary. The RH strain of *Toxoplasma gondii* has been propagated for 15 serial passages and a second strain for 7 passages in roller tube cultures of mouse embryonic tissues. Multiplication of the RH strain was also observed in roller cultures of human foreskin, uterus and embryonic skin-muscle tissues. In each instance, the appearance of organisms in the culture was associated with extensive degenerative changes and ultimate destruction of the tissue. At the point of apparent maximum release, the culture fluid contained large numbers of the protozoa.

We are indebted to Miss Helen M. Witton and Mrs. Lorraine B. Sanford for technical assistance and to Mr. John Carabitses for the preparation of the photomicrographs.

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1. Sabin, A. B., and Olitsky, P. K., *Science,* 1937, v85, 336.

2. Guimarães, F. N., and Meyer, H., *Rev. Bras. Biol.,* 1942, v2, 123.

3. Meyer, H., and Oliveira, M. X. de, *Rev. Bras. Biol.,* 1943, v3, 341.

4. ———, *ibid.,* 1945, v5, 145.

5. Mühlpfordt, H., *Z. Tropenmed. Parasit.,* 1952, v4, 53.

6. Jacobs, L., *Am. J. Trop. Med. and Hyg.,* 1953, v2, 365.

7. Lock, J. A., *Lancet,* 1953, v1, 324.

8. Robbins, F. C., Weller, T. H., and Enders, J. F., *J. Immunol.,* 1952, v69, 673.

9. Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 100.

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Epidemic Histoplasmosis and Airborne *Histoplasma capsulatum.* (20789)

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Several authors(1,2) have suggested that infection with *Histoplasma capsulatum* is acquired by the airborne route. This route of infection is supported by the primary localization in the lungs, the frequency of pulmonary involvement, the small size of the majority of the spores(3), and the occurrence of laboratory infections(4). This evidence is further supported by the recent report of a number of epidemics of histoplasmosis(5,6) where infections appeared to have been acquired from the air. *H. capsulatum* has been isolated from man, as well as from wild and domesticated animals, and from soils collected in various areas of the United States(6,7). The study reported in this paper represents the first isolation of the fungus from the atmosphere. An epidemic of histoplasmosis in a farm family near Atchison, Kansas, afforded an excellent opportunity to culture the air for the presence of *H. capsulatum* in a specific locality. The entire family of 5 became ill with an influenza-like illness about 10 days after cleaning an unused chicken house. One patient required hospitalization for 62 days. *H. capsulatum* was isolated from farm animals and the soil both inside and immediately outside of the chicken house. All 5 persons involved in the epidemic had pulmonary lesions demonstrable by X-ray and all of them had initially positive serological test for histoplasmosis, followed by falling titers at later examinations. A similar epidemiological picture was found near Beverly, Mo. A tenant farmer had cleaned an abandoned chicken house and contracted histoplasmosis as shown by X-ray and serological changes. The etiological agent was isolated from the debris in the chicken house.

Material and methods. The air in the chicken houses was sampled using the Venturi Scrubber air sampler. In this sampler, particles are removed from the air by the scrubbing action of the fluid spray produced by Venturi action. The fluid is recirculated by suction created at the Venturi constriction. The principles of the Venturi Scrubber have been adequately described(8). The apparatus is used industrially for the cleansing of air(9). Two sizes of scrubber samplers were used in these studies. The larger sampler has a Venturi tube with the following approximate dimensions: Length 13½ inches, diameter 2 inches, and the throat constriction ¾ of an inch. When the scrubber is in operation, the working volume of the liquor in the bowl is approximately 1270 ml. The smaller sampler has the following approximate dimensions: Length 4¼ inches, diameter ⅝ of an inch, and throat constriction ¼ of an inch. The scrubbing liquor in the bowl at working volume is approximately 36 ml. The larger sampler was used with a tank-type vacuum cleaner which pulled 16 cfm. of air through the sampler. The smaller sampler was used with a vacuum pump which pulled 2.5 cfm of air through the scrubber. The samplers were sterilized in the autoclave prior to use. Sterile distilled water was used in the samplers as the scrubbing liquor. At the completion of the sampling period, the scrubbing fluid from the air sampler was filtered through a sterile Goetz millipore filter to concentrate the solid material captured from the air. The sediment when scraped from the filter pads and resuspended in sterile saline. The suspension was treated with 8000 units each of penicillin and streptomycin per ml. The treated sample was stirred and permitted to stand undisturbed for 30 minutes. After shaking the sample, white Swiss mice were inoculated with one ml of the suspension intraperitoneally. The following 3 consecutive days each mouse received 10000 units of penicillin subcutaneously. Four weeks after inoculation the mice were sacrificed. The liver, spleen, and adrenals of each mouse were placed in a sterile mortar and macerated, using sterile saline as the diluent. The macerated tissues were cultured on one brain heart infusion plate with 10% blood and 2 Sabouraud's plates. These plates contained 40 units of streptomycin and 20 units of penicillin per ml of media. One ml of the tissue-saline suspension was inoculated onto each plate. The plates were incubated at room temperature for 4 weeks. Colonies were examined using lacto-phenol blue preparations. On all but one occasion the air sampling on the farms was limited to the interior of the chicken houses, and only the large Venturi Scrubbers were employed. These scrubbers were supported 2 or 5 feet off the ground floor. On the one occasion when the small Venturi Scrubber was used, it was supported 6 inches off the ground on the outside of the chicken house.

Results. Table I presents a summary of the results of air sampling at 2 sites.

Reference to Table I shows that positive samples were obtained from the Kansas site on only one day. Perhaps this is explained by the fact that on the previous day the chicken house was raised approximately 6 inches off the ground in preparation for moving it to another location on the farm. The chickens had been removed 4 months previously. Since the chicken house had no flooring, the dry straw and droppings covering the ground remained undisturbed. The intakes of the large Venturi samplers were faced east 2 feet off the ground floor. These samplers were in operation for 3 hours. The smaller sampler was assembled outide the chicken house on the north side 6 inches from the building. The intake of the sampler was faced south. It was 6 inches off the surface of the soil. A 30-minute sample of the air was taken at this point. The wind velocity was estimated at 10 to 12 miles per hour, but the air was not visibly dusty. The soil surface and debris on the floor of the chicken house were not disturbed during the sampling except by the southerly wind. Air sampling on this day, resulted in isolation of *H. capsulatum* from the air with both the large and small Venturi scrubbers.

The air sampling results obtained at the Missouri site are particularly significant be-

TABLE I. Summary of Results of Air Sampling.

Location	Date	Type Venturi used	Sampling time, hr (each sampler)	Ht of samples from ground, ft	No. of mice inoc.	No. mice died 48 hr and not cultured	No. pos. mice	% pos. mice
Kans. site	12/11/52	Large	1.5	5	7	6	0	0
	1/27/53	"	1.5	5	8	0	0	0
	2/18	"	.5	5	9	0	0	0
	3/13	"	1.5	5	7	0	0	0
	4/14	"	3.0*	2	35	5	11	37
	4/14	Small	.5	½	4	0	1	25
Mo. site	10/31/52	Large	1.5	2	7	4	0	0
	7/ 8/53	"	4.5*	2	5	0	0	0
	7/ 8	"	4.5	5	5	0	0	0
	7/17	"	4.0*	2	8	4	2	50
	7/17	"	4.0	5	5	1	2	50
	7/23	"	4.0†	2	7	0	0	0
	7/29	"	4.5†	2	7	0	7	100
	8/ 6	"	4.5*	2	7	0	3	43
Total					121	20	26	26

* Two samples were run simultaneously and fluids combined.
† Three " " " " " " .

cause *H. capsulatum* could be repeatedly isolated from the air in the chicken house. Considering the possibility of respiratory infection, it is especially important to note that on one occasion the intake of the sampler was 5 feet above the floor. The tenants cleaned the chicken house on July 26, 1953, removing planks that had been laid loosely on the ground. This cleaning did not alter the presence of *H. capsulatum* since the fungus was recovered before and after the cleaning of the building. During the 3 sampling periods when *H. capsulatum* was isolated the wind was calm and the debris on the flooring was not disturbed.

Summary. It is believed that these 5 isolations represent the first time that *H. capsulatum* has been obtained from naturally contaminated air. These results and the presence of an epidemic pulmonary disease in the residents on these farms provide evidence that *H. capsulatum* can be airborne. These results also show that the large and small Venturi scrubbers of the type employed are capable of removing the spores of *H. capsulatum* from air.

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1. White, F. Clark, and Hill, Harry E., *Am. Rev. Tuberc.*, 1950, v62, 1.
2. Grayston, J. T., Loosli, C. G., and Alexander, E. R., *Science*, 1951, v114, 323.
3. Cozad, George C., and Furcolow, Michael L., *J. Infect. Dis.*, 1953, v92, 77.
4. Furcolow, Michael L., Guntheroth, Warren G., and Willis, Myron J., *J. Lab. and Clin. Med.*, 1952, v40, 182.
5. Grayston, J. Thomas, and Furcolow, Michael L., *Am. J. Pub. Health*, 1953, v43, 665.
6. Furcolow, Michael L., and Grayston, J. Thomas, *Am. Rev. Tuberc.* 1953, v68, 307.
7. Emmons, C. W., *Am. J. Pub. Health*, 1950, v40.
8. Jones, W. P., *Indus. and Eng. Chem.*, 1949, v41, 2424.
9. Collins, T. T., Jr., Seaborne, C. R., and Anthony, A. W. Jr., *Paper Trade J.*, 1947, v124, 45.

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Studies on Oxygen Poisoning: Protective Effect of β-Mercaptoethylamine.*†
(20790)

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Based on theoretical considerations and analogies found in the literature, an hypothesis has been formulated(1) on a possible mechanism in common between the initial fundamental action of high oxygen pressures and the primary mechanism of x-radiation. The latter is generally considered to be mainly indirect and due to the oxidizing free radicals $OH^\bullet$ and $O_2H^\bullet$(2,3). When high oxygen pressure and x-rays were applied simultaneously to mice, a synergistic effect was revealed by a significant shortening of the survival time in the acute oxygen experiments(1).

This prompted the investigation of other possible manifestations pointing to a common path of action. Further evidence lending some support to the hypothesis is now accumulating. It was found that agents of different chemical nature known to give protective action against x-radiation lethality also prolonged significantly the survival times of our mice submitted to acute high oxygen pressure. This was the case for glutathione (4,5), cysteine(6,7), and ethanol(8) which also greatly protected against oxygen poisoning(1). Bacq *et al.*(9) showed a clear protective effect in x-irradiated mice pretreated with β-mercaptomethylamine and this was confirmed by Rugh and Wang(10). Experiments showing a similar effect in mice submitted to high oxygen pressure are presented below.

Methods. Forty "Webster" female mice of the Clara Lynch strain, weighing approximately 20 g each, were used. Two groups of 20 mice (10 controls and 10 experimentals) were tested on 2 different days. The 10 experimental mice received intraperitoneal injections of 3.33 mg of β-mercaptoethylamine in 0.2 ml of saline. (1.0 ml of Becaptan hydrochloride—S. A. Labaz—was diluted to 6.0 ml with saline.) The control mice received an equal volume of saline. Within 5 minutes after the injections the 10 control mice were put in one chamber and the 10 Becaptan-injected mice were put in the other. The 2 chambers were flushed for one-half minute with oxygen from a single cylinder fitted with a "Y" tube and then were brought directly to 6 atmospheres (75 ψ gauge) of oxygen pressure. During the experiment there was a continuous flow of oxygen sufficient to keep the carbon dioxide concentration in the chambers close to a zero level. Survival times were measured from the time that a pressure of 6 atmospheres was attained until the cessation of respirations.

Results. In the first experiment the survival times for the mice injected with β-mercaptoethylamine ranged from 51 to 84 minutes, the average being 66.1 minutes. The control mice survival times ranged from 37 to 53 minutes, the average being 44 minutes.

In the second experiment the survival times of the treated mice ranged from 70 to 102 minutes and of the controls from 37 to 54 minutes. The respective averages being 89 and 45.9 minutes.

The average survival time for both groups of treated animals was 76.9 ± 2.72 minutes and the control average was 45 ± 1.39 minutes. The actual times of death of the various animals are shown in Fig. 1.

Discussion. In the extensive work of Bacq *et al.* on the effect of β-mercaptoethylamine ($SH\text{–}Ch_2\text{–}CH_2\text{–}NH_2$ = Cysteamine = Becaptan R.N.), evidence was produced show-

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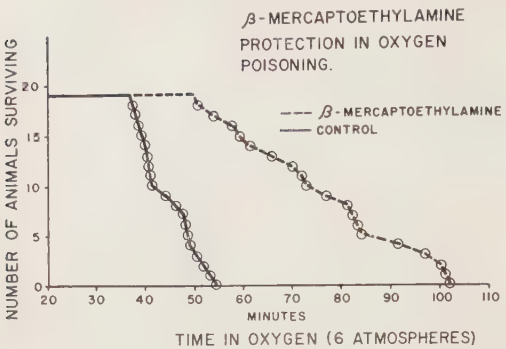

FIG. 1.

ing that this substance exerts a marked protective action against the effects of irradiation on mice and on certain other biological systems. A discussion of the biological actions of β-mercaptoethylamine can be found in the paper by Bacq *et al.*(9). This substance is not only interesting because of its –SH group, which might account for its protective action, but also because it has been shown to be a part of Coenzyme A(11-13). In this connection Bacq and Herve(14) have found that Coenzyme A, in equimolecular concentrations, seems to be much more active than β-mercaptoethylamine as a protector against x-rays. On the other hand, Stadie *et al.*(15), Mann and Quastel(16), and Dickens(17) have studied a number of –SH enzymes showing different degrees of sensitivity to high oxygen tensions. Succinic dehydrogenase, among others, was inactivated by increased oxygen tensions, but the enzyme system showing the greatest sensitivity was the pyruvic oxidase system. Dickens(17) working with brain tissues, suggested that the primary effect of oxygen poisoning may be an inactivation of the pyruvic oxidase system, although possible effects on other enzyme systems were not excluded. The *in vitro* inactivation of –SH containing and other enzymes by ionizing radiations has been well demonstrated by Barron *et al.*(18). It has been claimed both for oxygen poisoning(19) and irradiation(20) that *in vivo* there is no appreciable change in the activity of the oxidative enzyme systems studied and no significant change in oxygen uptakes, to explain the deleterious effects produced by these agents. However, oxygen uptake may not always be a good index of a normal metabolic ability of the cell(21).

Among the many factors contributing to the sum of biochemical lesions resulting in the symptoms produced by oxygen poisoning or by x-radiation, the role of changes produced in fatty acid metabolism(22,23) may prove to deserve consideration.

If one chooses to think that the protective effect shown by β-mercaptoethylamine is due to its role as a component of Coenzyme A, (a coenzyme for the pyruvic oxidase system) and if pyruvic acid is regarded as linking the metabolism of carbohydrate, fat, and protein, then a general metabolic breakdown may be pictured when there is interference with the normal oxidative decarboxylation of pyruvic acid. In the above mentioned experiments of Bacq and Herve(14) protection against x-irradiation was obtained with 3 mg of Pabst Coenzyme A per 20 g mouse, injected intraperitoneally, this being equivalent to approximately 0.3 mg of β-mercaptoethylamine on a molecular basis. In order to find out if Coenzyme A would also protect against oxygen poisoning, we used the same dose and site of injection, and have obtained in a few experiments a highly significant protection.

With Coenzyme A (Pabst), Lot No. 402, in 2 experiments with a total of 9 treated and 11 control mice we obtained 26.4 minutes of protection which was significant at the 0.3% level. However, with Coenzyme A from Lot No. 403 in 4 experiments with 30 treated and 30 control mice, we obtained only 8.0 minutes of protection, significant at a 2.6% level. Because there was a consistent difference in the protection with each lot, we plan to do further oxygen poisoning experiments with Coenzyme A. Since Coenzyme A is a coenzyme for a number of enzymatic systems other than the pyruvic oxidase system, it would be well to keep in mind the possibility that x-rays and high oxygen pressure might affect any of these systems through a primary action on Coenzyme A.

The possible applications of this substance to deep sea diving and retrolental fibroplasia problems are now under study.

Conclusions. Mice exposed to 6 atmospheres of oxygen are protected and their survival prolonged 70% by previous intraperitoneal injection of 3.33 mg of β-mercaptoethylamine per 20 g body weight.

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1. Gerschman, R., Gilbert, D. L., Nye, S. W., Dwyer, P., and Fenn, W. O., *Science*, in press.

2. Sixth International Congress of Radiology, *Brit. J. Radiol.*, 1951, v24, 416, 422, and 428.

3. Barron, E. S. G., Seki, L., and Johnson, P., *Arch. Biochem. Biophys.*, 1952, v41, 188.

4. Chapman, W. H., Sipe, C. R., Eltzholtz, D. C., Cronkite, E. P., and Chambers, F. W., *Radiology*, 1950, v55, 865.

5. Chapman, W. H., and Cronkite, E. P., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 318.

6. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, 1949, v110, 213.

7. Patt, H. M., Smith, D. E., Tyree, E. B., and Straube, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 18.

8. Cole, L. J., and Ellis, M. E., *Am. J. Physiol.*, 1952, v170, 3.

9. Bacq, Z. M., Dechamps, G., Fischer, P., Hervé, A., LeBihan, H., Lecomte, J., Pirotte, M., and Rayet, P., *Science*, 1953, v117, 633.

10. Rugh, R., and Wang, S. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 411.

11. Snell, E. E., Brown, G. M., Peters, V. J., Craig, J. A., Wittle, E. L., Moore, J. A., McGlohon, V. M., and Bird, O. D., *J. Am. Chem. Soc.*, 1950, v72, 5349.

12. Baddiley, J., and Thain, E. M., *J. Chem. Soc.*, 1951, 2253.

13. Gregory, J. D., and Lipman, F., *J. Am. Chem. Soc.*, 1952, v74, 4017.

14. Bacq, Z. M., and Hervé, A., *Arch. Internat. de Physiol.*, 1953, v61, 434.

15. Stadie, W. C., *et al., J. Biol. Chem.*, 1945, v161, 153, 175, 181, 189.

16. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, 1946, v40, 139.

17. Dickens, F., *Biochem. J.*, 1946, v40, 145 and 171.

18. Barron, E. S. G., Dickman, S., Muntz, J. A., and Singer, T. P., *J. Gen. Physiol.*, 1949, v32, 537.

19. Stadie, W. C., and Haugaard, N., *J. Biol. Chem.*, 1946, v164, 257.

20. Patt, H. M., *Physiol. Rev.*, 1953, v33, 35.

21. Dawson, R. M. C., *Biochem. J.*, 1953, v55, 507.

22. Penrod, K. E., *Fed. Proc.*, 1953, v12, 108.

23. Mead, J. F., *Science*, 1952, v115, 470.

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Excretion of Carbon-14 by Man after Administration of Morphine-N-Methyl-C^{14}.*† (20791)

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In contrast to the considerable information available concerning the excretion of morphine in the human addict(1-3), little attention has been directed to morphine excretion in non-addicted man following administration of a single therapeutic dose. Oberst has presented data on urinary concentration of free and bound morphine in normal man but figures for complete excretion are lacking(3). Thus most of our present knowledge regarding morphine excretion in the normal animal has been derived from data on dogs(4-6) and rats(7). In both these species, it has been shown that with the development of tolerance there is a concomitant alteration in the excretion of morphine metabolites as evidenced by a marked reduction in the recovery of bound morphine from urine. Although it is possible that a parallel alteration occurs in man following the development of addiction, nevertheless, precise data on normal man are desirable. In view of the relatively small amount of morphine contained in a therepeutic dose, it was felt that the application of tracer technics to this problem would be helpful.

Experiments on rats using N-$C^{14}H_3$ labeled morphine indicated that excretion of radio-

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activity is virtually complete in 48 hours(8). This suggested that excretion might be rapid enough in man to warrant the injection of radioactive morphine. Since only limited amounts of carbon-14 can be administered to normal humans, the present study was initiated to determine the feasibility of doing tracer studies in normal humans using one microcurie or less of radioactive carbon and as a preliminary step toward isolation and identification of metabolic products of morphine. This report deals with the quantitative aspects and time relations involved in the urinary, fecal and pulmonary excretion of carbon-14 by cancer patients and normal human subjects following the administration of N-$C^{14}H_3$ labeled morphine contained in a single therapeutic dose.

Methods. The morphine-N-methyl-C^{14} used in these studies was synthesized by Rapoport, Lovell and Tolbert(9). It was converted to the sulfate and diluted with commercial morphine sulfate for intramuscular injection. The cancer patients received a dose of 15 mg containing 17.2 microcuries of carbon-14. The normal subjects were given 10 mg containing a total of 0.9 microcuries of carbon-14. The subjects included 3 terminal cancer patients and 2 normal adult volunteers as follows: A. C., ♀, age 49, carcinoma of the colon with metastases; H. B., ♂, age 68, multiple myeloma; H. E., ♂, age 32, normal; P. R., ♀ age 33, normal. The cancer patients were not markedly debilitated and had no gross disturbance of liver, kidney, or bowel function with the exception of A. C. who had a persistent colitis partially controlled by terramycin. A. C. and H. B. received no morphine type analgetics for 48 hours prior to radio-morphine injection. In addition to these non-addicted subjects, one cancer patient designated as a morphine addict was included in this study. This patient, V. M., ♀, age 31 with carcinoma of the colon with metastasis, had been taking morphine or similar analgetics for 2 years and at the time of this experiment had been taking daily oral doses of up to 90 mg of morphine.

One of two methods was used for determination of respiratory $C^{14}O_2$ depending on the amount of radioactivity administered. For the cancer patients it was sufficient to have the subject rebreathe for 1 minute into a 9 liter rubber bag containing about 6 liters of oxygen. This CO_2 thus collected was absorbed in a NaOH tower and subsequently precipitated as $BaCO_3$ which was weighed to determine the per minute output of CO_2. Samples with a specific activity of greater than 10 dis./min./mg $BaCO_3$ were counted with a proportional counter (Nucleometer, Radiation Counter Laboratory, Chicago, Ill.), others were converted to CO_2 and measured in an ionization chamber. The instruments were calibrated with samples prepared from standard carbon-14 obtained from the National Bureau of Standards, Washington, D. C. Breath samples from the normal adults used in this study were obtained by having the subjects breathe directly into a vacuum line designed for the collection and purification of CO_2(10). Three- to 6-minute breath samples were collected and stored in one liter bulbs. Radioactivity was then measured in a 500 cc ionization chamber using the rate of drift method with a vibrating reed electrometer (Applied Physics Corp., Pasadena, Calif.) and a continuous recorder (Brown Potentiometer Recorder, Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.) The sensitivity of this method was about 0.004 dis./min./mg $BaCO_3$. *Urine samples* were collected at varying intervals for 3 days following drug injection. Aliquots were dried and combusted to CO_2 using a chromic-sulfuric acid oxidizing mixture(11). Feces were collected for 3 days and aliquots of each 24-hour specimen combusted as above. The CO_2 obtained from urine and feces combustions was precipitated as $BaCO_3$ and radioactivity measured using 100 cc ionization chambers.

Results. The output of $C^{14}O_2$ in the breath was estimated by plotting the total radioactivity obtained from each breath sample versus time and mechanically integrating the rate plot thus obtained. The area under the curve was cut into strips corresponding to one or two hours on the time axis and the weight of each compared with the weight of a section of the graph paper which was equivalent to a convenient number of disintegrations per minute. The peak rate of $C^{14}O_2$ excretion

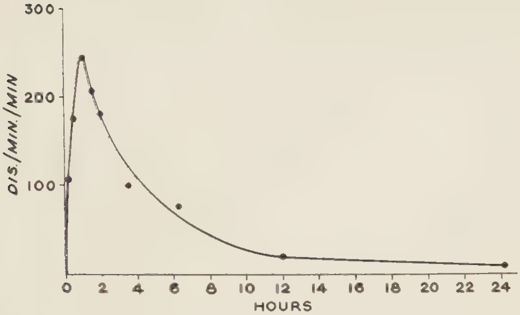

FIG. 1. Typical rate plot of pulmonary $C^{14}O_2$ excretion (subject H.E.).

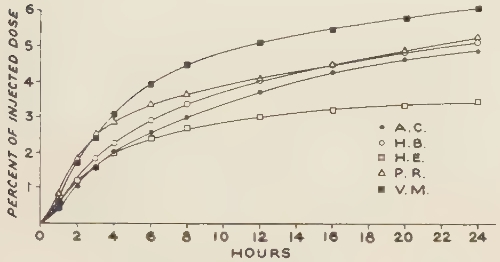

FIG. 2. Pulmonary excretion of $C^{14}O_2$ for the 24 hr period following the administration of labeled morphine.

in the breath occurs between 30 and 90 minutes after drug administration, corresponding to the time when the pharmacologic actions of morphine are most profound (see Fig. 1), and then falls off rapidly. The cumulative 24-hour pulmonary $C^{14}O_2$ excretion is shown for all subjects in Fig. 2. The 24-hour pulmonary excretion of carbon-14 ranged between 3.4 and 5.3% for the nontolerant subjects. Although more pulmonary $C^{14}O_2$, 6.1%, was recovered from the addict (V.M.) further studies are required to determine whether this represents a significant difference.

Measurable amounts of radioactivity were present in breath samples obtained from H.B. 5 days after injection and from V.M. 4 days but not 8 days after injection. This suggests that to a limited extent a transfer of $C^{14}H_3$ groups from the morphine molecule to the metabolic pool may occur from which $C^{14}O_2$ is slowly liberated by normal catabolic processes. In the metabolism of glycine in man (12,13) approximately 40% of the methylene carbon is converted to CO_2 in 24 hours. If one assumes that the rate of metabolism of the N-methyl group of morphine and the N-methylene group of glycine are comparable then one can estimate that the 5% pulmonary excretion of $C^{14}O_2$ in these subjects represents about 12.5% breakdown of the morphine to produce methyl groups for general body metabolic processes. There is also the possibility that a small amount of the intact morphine molecule is held in some firm combination which is slowly metabolized, since Gross and Thompson(4) have shown that the nontolerant dog continues to excrete small amounts of morphine for at least 6 days. It should be further noted that an interesting species difference between rat and man exists in the pulmonary excretion of radioactivity. The sex difference which occurs in the rat(8) was not found in our studies on humans.

Considerable differences are observed in total urinary excretion of radioactivity (Fig. 3) which may be related in part to urine output. The addict (V.M.) maintained a high urinary output during the entire course of the experiment and exhibited a marked diuresis for the first 6 hours during which her rate of Carbon-14 excretion was well above that of the other subjects. Two subjects, A.C. and P.R., had low urine outputs for the first 6 hours with correspondingly low carbon-14 outputs. A similar coupled trend was observed by Plant and Pierce(14) in their studies on dogs and by Oberst(1) in his studies on human addicts. Appreciable amounts of activity were eliminated in the feces of non tolerant subjects and, in contrast to urinary excretion, the amounts are fairly

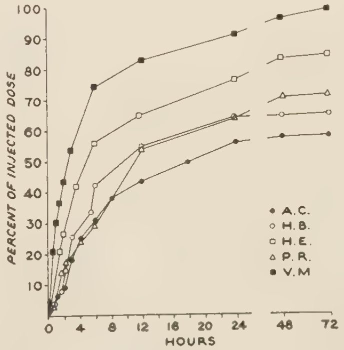

FIG. 3. Urinary excretion of carbon-14 following administration of labeled morphine.

TABLE I. Fecal Excretion of Carbon-14 as Related to Feces Output.

		Non-addict				Addict
Hr		A.C.	H.B.	H.E.	P.R.	V.M.
0-24	g feces	412	109	159	0	0
	% dose	6.91	2.24	7.34	0	0
24-48	g feces	375	151	170	443	123
	% dose	.27	4.22	1.60	9.46	.04
48-72	g feces	353	22	350	0	0
	% dose	.06	.27	1.44	0	0
72-96	g feces	—	—	—	74	49
	% dose	—	—	—	.88	.03
Total	g feces	1140	282	679	517	172
	% dose	7.24	6.73	10.38	10.34	.07

TABLE II. Summary of Carbon-14 Recovery from All Sources.

	Non-addict				Addict
Subject	A.C.	H.B.	H.E.	P.R.	V.M.
Urine	57.5	64.7	83.7	71.1	98.3
Feces	7.2	6.7	10.4	10.3	.1
Breath	4.9	5.2	3.4	5.3	6.1
Blood	.2	.1	—	—	—
Vomiting & duodenal drainage	.7	—	—	—	—
Total	70.5	76.7	97.5	86.7	104.5

TABLE III. Concentration of Carbon-14 in Duodenal Secretions of Subject A.C.

Hr	.5	1.5	3.0	6.0	12.0	24.0
Dis./min./ml	391	508	5469	10360	3670	4760

constant (Table I). Fecal excretion of carbon-14 by the addict V.M. was negligible and since this represents a significant difference from the nontolerant subjects it should be further studied.

Total carbon-14 recoveries from all sources ranged from 71-105% (Table II). The highest values were obtained on those subjects who had high urinary carbon-14 outputs, V.M. and H.E., and in these the pulmonary excretion of $C^{14}O_2$ was already quite slow at the end of 24 hours. Since the slope of the pulmonary excretion curve (Fig. 2) for the other three subjects indicates that there is continued production of $C^{14}O_2$ at the end of 24 hours, one can postulate that there is here a more extensive incorporation of the N-methyl group of the morphine molecule into the general body carbon pool. This could account in part for the low radioactivity recovery in these cases. To determine the role of the intestinal tract in the disposition of morphine, a duodenal tube was passed in subject A.C. and samples obtained at intervals for 24 hours. The concentration of carbon-14 in duodenal secretions at various times is shown in Table III. Assuming that duodenal carbon-14 originates in the biliary excretion and that the 24-hour bile output is 500 ml (15), integration of the curve obtained by plotting concentration versus time (Table III) gives a total of 7.4% of the injected dose potentially available for excretion via the intestinal route. Since this figure closely approximates the experimentally determined figure of 6.9% for the 24-hour fecal excretion the inference may be drawn that the total fecal output arises from bilary excretion. The reabsorption of this material from the intestines is apparently negligible, since 24-hour retention of feces by subject P.R. did not result in a decreased fecal carbon-14 output as compared with other nontolerant subjects.

Previous work by Oberst(3) has shown that morphine appears in the bile in conjugated form but is recovered from the feces in free form only. It would appear from our results, therefore, that hydrolysis of conjugated morphine must take place in the intestine. This is supported by the finding that the intestinal flora of the rat are able to hydrolyze glucuronides(16). It is of interest to note that a striking species difference exists between rat and man in the disposition of morphine as evidenced by a 5-fold greater fecal excretion and at least a 9-fold greater biliary excretion in the rat(8). It would appear that in the rat much intestinal morphine of biliary origin is subsequently reabsorbed.

Summary. 1. Therapeutic doses of morphine-N-methyl-C^{14} have been administered to 5 human subjects. The major portion of the radioactivity is excreted in 24 hours. Urinary excretion accounted for most of the recovered activity, and there was some correlation between urine volume and carbon-14 excretion when these volumes were abnormally high or low. 2. Fecal excretion accounted for 7 to 10% of the dose and the possibility that this material originates in the bile as "bound" morphine with subsequent hydrolysis in the intestine is discussed. Intestinal excretion is not as important in man as in rats. 3. Pulmonary excretion of $C^{14}O_2$ ranged from 3½ to 6% of the injected dose in 24 hours. There was no sex difference in excretion by this route, as has been observed in the rat. 4. The carbon-14 excretion pattern for the one addict was similar to the nontolerant subjects, except in the case of the feces. 5. It has been shown that it is feasible to do tracer studies in normal subjects using less than a microcurie of carbon-14.

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1. Oberst, F. W., *J. Pharmacol. and Exp. Therap.*, 1940, v69, 240.
2. *Idem*, 1941, v73, 401.
3. *Idem*, 1942, v74, 37.
4. Gross, E. G., and Thompson, V., *J. Pharmacol. and Exp. Therap.*, 1940, v68, 413.
5. Thompson, V., and Gross, E. G., *ibid.*, 1941, v72, 138.
6. Gross, E. G., PROC. SOC. EXP. BIOL. AND MED., 1942, v51, 61.
7. Zauder, H. L., *J. Pharmacol. and Exp. Therap.*, 1952, v104, 11.
8. March, C. H., and Elliott, H. W., *Fed. Proc.*, 1952, v11, 373.
9. Rapoport, H., Lovell, C. H., and Tolbert, B. M., *J. Am. Chem. Soc.*, 1951, v73, 5900.
10. Baker, E. M., Marcus, M., and Tolbert, B. M., to be published.
11. Skipper, H. E., Bryan, C. E., White, L. Jr., and Hutchinson, O. S., *J. Biol. Chem.*, 1948, v173, 371.
12. Berlin, N. I., Tolbert, B. M., and Lee, H. C., *J. Clin. Invest.*, 1953, v32, 1.
13. Berlin, N. I., Tolbert, B. M., and Lawrence, J. H., *ibid.*, 1951, v30, 73.
14. Pierce, I. H., and Plant, O. H., *J. Pharmacol. and Exp. Therap.*, 1932, v46, 201.
15. Gamble, J. L., *Extracellular Fluid*, Cambridge, Mass., 1950 Chart 37.
16. Glazko, A. L., Dill, W. A., and Wolf, L. M., *J. Pharmacol. and Exp. Therap.* 1952, v104, 452.

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Effect of Restraint on Temperature Regulation in the Cat.* (20792)

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Hypothermia produced by restraint in mice has been previously reported(1), and a brief review of the literature dealing with this subject presented. To the authors' knowledge only the smaller thermolabile laboratory animals have been studied for this response. This group includes the rabbit with thermal instability commonly noted in routine work (2). In the present series, cats, generally thermostable animals, have been stressed by restraint and the effect on body temperature noted. It is known that stresses, which are partially emotional in nature, result in a loss of temperature regulation of smaller mammals, with a resultant body temperature drop when the animals are exposed to cold(1). If such a response to stress, either emotional or physical, existed in man, the lowering of body temperature in the cold would impose a serious handicap on such individuals as mili-

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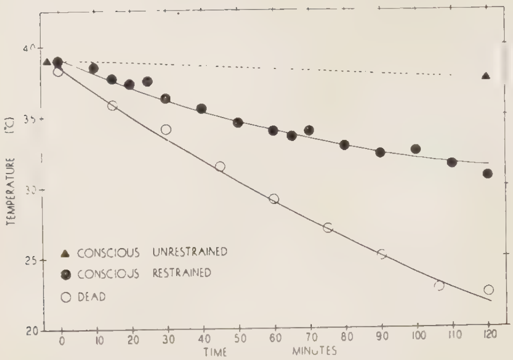

FIG. 1. Comparison of avg temperature drop of dead cats, restrained cats, and unrestrained cats (controls) in the cold (–15°C ± 2°).

tary personnel who must perform at peak efficiency. Thus the necessity of studying this problem in a large thermostable animal.

Methods and materials. Forty adult mongrel cats of either sex were used. They were divided into 14 controls, 13 restrained, and 13 dead animals. The control animals were housed individually in large cages in a cold room (–15°C ± 2°C). Deep rectal temperatures were taken with a thermometer at the beginning and end of the tests. The restrained animals were treated in one of two manners. Some had their legs, placed in normal positions, tied to 4 uprights; others, still in a normal position, had their hind legs tied to pegs while their neck was placed between uprights too close together for the head to be withdrawn. In both of these categories, the animals were placed in the cold room and body temperatures were taken intermittently with indwelling rectal thermometers inserted to a depth of 3 to 5 inches. The indwelling thermometer should be considered a part of the stressing technic. The third group of cats was killed by an overdose of sodium pentobarbital, placed in the cold room with indwelling rectal thermometers and temperatures taken intermittently throughout the duration of exposure.

Results. As shown in Fig. 1, the restrained animals lost body temperature (terminal temp. 31.5°C) while the control animals housed in large cages maintained an essentially normal body temperature (37.5°C). The body temperature of the restrained animals dropped in spite of continuous struggling which was maintained throughout the entire length of the exposure. A comparison of the curve for body temperature loss in the restrained cats with that for the dead cats shows that the restrained cats lost body temperature much more slowly than the dead animals (terminal temp. 22°C).

Discussion. Although several investigators (1-4) in working with rats, mice, or rabbits, have reported restraint-induced hypothermia, there has, to the authors' knowledge, been no report of such a phenomenon in a large, thermostable laboratory animal such as the cat. Most of these investigators used a modification of the Hamilton technic(3) and stated the belief that emotionality was an important factor contributing to hypothermia. Ware *et al.*(5) have reported they were unable to obtain hypothermia by use of this method unless the restraining cage was tight enough to restrict breathing. This work raised serious questions concerning an "emotional" nature of the stress and caused many to doubt the reliability of the conclusions drawn by other investigators using this technic. The cats used in the present studies were not caged and therefore there was little possibility of restraint of breathing. Body temperature fell, nevertheless, thus indicating that hypothermia can be produced in the cat by restraint without restriction of breathing. As mentioned previously, the restrained animals, without exception, struggled vigorously during the exposure. For body temperature to fall under such conditions in an otherwise relatively thermostable organism, distinct physiological changes favoring heat dissipation must have occurred. The possibility of an emotional factor and the physiological mechanism involved are being studied. A comparison of the curves for body temperature drop in the dead and restrained animals indicates that the restrained cats maintained temperature regulation better than mice under similar conditions(1).

The fact that a stressful situation so dramatically interferes with temperature regulation in the cat suggests that a similar mechanism may exist in the human. Thus the problem of proper morale and indoctrination becomes more acute in light of the possible effects of such an induced hypothermia upon personnel exposed to a stressful situation in the cold.

Summary. Healthy adult male and female cats were restrained in essentially normal positions by binding of legs or head to stationary supports. The effect of exposure on the body temperature of these animals in a cold room (–15°C ± 2°C) was compared with that of dead animals and with controls which were housed individually in large cages. Temperatures were taken with indwelling rectal thermometers in the dead and restrained animals and at the beginning and end of the tests in the case of the control cats. Body temperatures of the restrained animals over a 2-hour exposure fell markedly (5.6°C) as compared to the control group (1.2°C). The temperature fall of the dead animals was the greatest (10.2°C). Since the animals were not caged, the hypothermia which resulted must be attributed to factors other than restricted breathing, perhaps partially to emotional stress.

1. Bartlett, R. G., Helmendach, R. H., and Bohr, V. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 4.
2. Sinelnikoff, E. J., *Arch. f. d. ges. Physiol.*, 1929, v221, 549.
3. Hamilton, J. B., *Am. J. Physiol.*, 1937, v118, 71.
4. Grant, R., *ibid.*, 1950, v160, 285.
5. Ware, A. G., Hill, R. M., and Schultz, F. H., *ibid.*, 1947, v149, 657.

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Effect of Fluoroacetate Upon Poliomyelitis in Monkeys.* (20793)

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Recent studies from this laboratory have demonstrated that the intraperitoneal administration of sodium fluoroacetate inhibits the growth of influenza virus in the lungs of mice (1) and that it suppresses the growth of the Lansing strain of poliomyelitis virus in the brains of mice(2). In the latter report it was shown that this compound also effected an increase in the incubation period of disease. These experiments as well as others employing tissue cultures(3) demonstrated that fluoroacetate does not destroy the virus upon simple contact but rather that its effect is obtained by interrupting the Krebs cycle so as to interfere with the utilization of citric acid. The accumulation of citric acid in large quantities in animal tissues following fluoroacetate administration has been adequately demonstrated(4-7) and its continued synthesis without further metabolism appears to be due to inactivation of the enzyme system necessary for the oxidation of citrate. The present report describes the effect of intravenously inoculated fluoroacetate upon cynomolgus monkeys and their infection with poliomyelitis virus.

Methods. Cynomolgus monkeys (*Macaca irus*) weighing between 1.5 and 3.5 kg were used throughout this study. A solution of sodium fluoroacetate was freshly prepared in distilled water and inoculated intravenously in measured quantities to give exactly the desired mg per kilo of body weight. Toxicity tests determined that a dosage of 5 mg per kilo was very near the maximum tolerated dose but was well tolerated by the animals except for occasional and temporary weakness, irritability, and anorexia. The citrate content of the brain tissue in 3 monkeys following administration of this dose of the drug was determined by the pentabromoacetone method(7). The values in γ/g wet tissue were found to be 33, 29, and 36, respectively. Normal values of 40, 41, and 42 obtained with untreated monkeys demonstrate that the brain is not the site of citrate accumulation. The significance of this finding will be considered in the discussion. In the experiments to be

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* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Effect of Fluoroacetate upon Infection of Cynomolgus Monkeys with 100 PD$_{50}$ Poliomyelitis Virus.

	Par	Not par	P	Onset paralysis (days)	Avg onset
				Treated (0 day)	
	3	2	.0952	8, 9, 13, 0, 0	10
	4	1	.0522	9, 11, 14, 17, 0	12.75
	3	1	.0216	8, 11, 18, 0	12.3
	7	1	.0192	5, 9, 10, 12, 17, 17, 19, 0	12.7
	8	2	.0043	8, 8, 8, 9, 10, 10, 12, 14, 0, 0	9.9
	8*	2	.0010	9, 9, 9, 11, 11, 12, 14, 14, 0, 0	11.1
Total	33	9			11.3
%	78.5	21.5			
				Treated (+3 day)	
	5	0		9, 11, 11, 16, 16	12.6
	5	0		7, 9, 11, 11, 11	9.8
	2	1	.4063	12, 12, 0	12
	6	4	.0129	6, 7, 7, 8, 9, 10, 0, 0, 0, 0	7.8
Total	18	5			10.1
%	78.2	21.8			
				Controls	
	10	0		5, 7, 7, 7, 8, 9, 9, 9, 11, 11	8.3
	5	0		7, 7, 8, 11, 11	8.8
	5	0		6, 6, 9, 11, 21	10.6
	4	0		4, 13, 16, 17	12.5
	10	0		7, 9, 9, 9, 9, 9, 10, 10, 10, 12	9.4
	10	0		7, 8, 8, 8, 10, 10, 14, 14, 17, 19	11.5
Total	44	0			9.9
%	100	0			

* 10 PD$_{50}$.

described, a dose of 5 mg/kilo was administered once either on the day of virus inoculation (0 days) or 3 days after virus (+3 days). The virus was the original Mahoney strain which had been isolated in this laboratory and adapted to subcutaneous invasiveness by repeated passage via this route. It was preserved as titrated pools in glass sealed ampoules in the frozen state until used. With the exception of one experiment which employed a 10^{-3} dilution, all inoculations consisted of one ml of a 10^{-2} dilution which represented 100 PD$_{50}$ of virus when given subcutaneously between the scapulae. Appropriate numbers of control monkeys were inoculated with virus alone and the animals were observed daily for clinical signs of illness. All questionable diagnoses were clarified by histological examination.

Results. The results of 6 experiments are summarized in Table I. All monkeys inoculated with virus alone developed paralysis. This result among the control animals emphasizes the adequacy of the viral dosage employed in these tests. In contrast, 9 of 42 monkeys (21.5%) which received 5 mg of fluoroacetate per kilo on the day of virus resisted the clinical infection as did 5 of 23 animals (21.8%) receiving the drug on the third day after virus.

The probabilities of these results occurring by chance were calculated by use of exact probability for a specific combination.† These probabilities for the 0 day treated groups are listed in Table I. From these it will be seen that the likelihood of chance occurrence of the results after 2 experiments was 5 in 100, and after 3 experiments as 2 in 100. Although the survival rate for all 6 experiments was calculated as being very significant (1 chance in 1000) the last 3 experiments were actually unnecessary since significance had been reached much earlier. This illustrates the advantage of calculating significance more frequently during the course of such experimental work. In contrast to the "0 day" results, significance was not reached until the fourth and final experiment when drug was administered on the third day after virus, although the per cent of survival was remarkably similar.

The average incubation period in drug-treated monkeys which developed paralysis was not significantly different from that of the control group.

Discussion. The suppression of viral growth in isolated tissues cultured outside the intact host is readily accomplished with a wide variety of metabolic inhibitors. Although these experiments provide valuable information concerning the growth requirements of a given virus such compounds are

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† The equation is $P = (r_1 ! r_2 ! C_1 ! C_2 !)/(a! b! c! d! N!)$ where the letters are taken from the form:

a	b	r_1
c	d	r_2
c_1	c_2	N

We are indebted to Dr. F. M. Hemphill for his assistance with the calculations.

rarely effective in the intact animal where chemical interference with virus is usually accompanied by the inhibition of some metabolic process essential to the well being of the host. Although efforts to affect the infection of monkeys with Brunhilde type poliomyelitis have been discouraging, some success has been achieved against the Lansing strain of poliomyelitis virus in mice(2,8-11). These results suggest that where survival is the criterion of effect, some advantage is gained in experimental chemotherapy with inhibitors which act upon the essential cellular metabolism. While the primary effect of fluoroacetate is a blockage of the oxidation of citric acid, the secondary effect is probably a temporary depletion of certain free amino acids of the cells which are diverted to citrate formation(12). It is through these secondary effects that one would expect viral development to be influenced(13). Since the duration of the effect of fluoroacetate in animals is short, and since in the present studies it was administered several days before virus could reach the central nervous system, its influence was probably directed upon some extraneural tissue which was important in the initiation of the infection. Furthermore, the data regarding citrate accumulation indicate that the levels of fluoroacetate employed in these experiments did not affect the brain. This finding concurs with more extensive studies(5,6) in other laboratories which have shown that the primary and secondary effects of fluoroacetate are not uniform in all tissues of the same species or in different species(14). This is in direct opposition to the conclusions on this subject reached by Mogabgab and Horsfall(15) who assumed that all tissues respond uniformly to the drug and concluded that the oxidation of citrate was a reaction unimportant to the development of influenza virus. Additional evidence of an extraneural effect of fluoroacetate is seen in the finding that the period of viremia is markedly reduced in monkeys treated with this compound(16). More extensive examination of tissues affected by fluoroacetate should reveal vital information of the pathways and sites of viral multiplication.

Summary. 1. Cynomolgus monkeys were treated with 5 mg/kilo of sodium monofluoroacetate on the day of, or 3 days following, subcutaneous inoculation of Type 1 poliomyelitis virus. 2. Nine of 42 monkeys which received drug on the day of virus resisted clinical infection as did 5 of 23 animals receiving drug on the third day after virus. All control animals became paralyzed. 3. Citrate did not accumulate in brain tissue of drug-treated monkeys suggesting that the action of fluoroacetate was at some site other than the brain. 4. The significance of these findings is discussed.

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1. Ackermann, W. W., *J. Exp. Med.*, 1951, v93, 635.
2. Ainslie, J. D., *ibid.*, 1952, v95, 9.
3. Brown, G. C., *J. Immunol.*, 1952, v69, 441.
4. Buffa, P., and Peters, R. A., *Nature*, 1949, v163, 914.
5. Potter, V. R., and Busch, H., *Cancer Res.*, 1950, v10, 353.
6. Potter, V. R., Busch, H., and Bothwell, J., Proc. Soc. Exp. Biol. and Med., 1951, v76, 38.
7. Kandel, A., and Chenoweth, M. B., *J. Pharm., and Exp. Therap.*, 1952, v104, 234.
8. Davies, W. L., Pond, W. L., Smith, S. C., Rasmussen, A. F., Jr., Elvehjem, C. A., and Clark, P. F., *J. Bact.*, 1952, v64, 571.
9. Gershoff, S. N., Rasmussen, A. F., Jr., Elvehjem, C. A., and Clark, P. F., Proc. Soc. Exp. Biol. and Med., 1952, v81, 484.
10. Wooley, J. G., Bond, H. W., and Perrine, T. D., *J. Immunol.*, 1952, v68, 621.
11. Brown, G. C., Craig, D.E., and Kandel, A., Proc. Soc. Exp. Biol. and Med., 1953, v83, 408.
12. Awapara, J., *J. Biol. Chem.*, 1952, v197, 695.
13. Ackermann, W. W., and Johnson, R. B., *J. Exp. Med.*, 1953, v97, 315.
14. Chenoweth, M. B., *Pharm. Rev.*, 1949, v1, 383.
15. Mogabgab, W. J., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1952, v96, 531.
16. Francis, T. F., Jr., Brown, G. C., and Kandel, A., unpublished results.

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Coproporphyrin Studies in Children. I. Urinary Coproporphyrin Excretion in Normal Children.* (20794)

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As a result of studies by Watson and associates(1), urinary coproporphyrin excretion has been found to be abnormal in many clinical conditions. In children, it is elevated, not only in congenital porphyria(2-4), but also in liver disease(5-7), lead poisoning (8-10), poliomyelitis(11), and blood dyscracias(12). Many of the disturbances of blood formation and hemoglobin synthesis seen characteristically in this age group will probably ultimately be found to be related to change in coproporphyrin metabolism(13). Schwartz and coworkers(14) have presented an improved method for measuring urinary coproporphyrins. By modifications including: (a) maintenance of a neutral or alkaline urine during the period of collection, (b) conversion and measurement of coproporphyrin precursor, (c) more efficient extraction, and (d) more efficient fluorimetry, a 3-fold increase of the average 24-hour value has been obtained by the new method as compared with the former "long method"(15). Zieve and his coworkers(16) have found that the mean coproporphyrin excretion for a group of 685 normal men was 189 ± 59 μg per day. There appeared to be a sequential increase in average coproporphyrin excretion as the customary alcohol intake increased in these men.

The purpose of the present report is to define the limits of 24-hour urinary coproporphyrin excretion in normal children as determined by the improved procedure. Subsequent reports will show the changes of urinary coproporphyrin excretion in various diseases in children.

Methods and materials. The main group of subjects studied consisted of 94 normal boys aged 6 to 16. All were residents in a summer camp for boys during the month of July. All were pronounced healthy by physical examination at the time of study and none had any history of serious illnesses in the past. All of the urines were collected over the same 24-hour period while the subjects were on the same diet and pursuing approximately the same amount of activity. None of the subjects consumed alcohol. Two other groups of subjects were studied in essentially the same manner: (1) normal adults, and (2) normal children under 6 years of age. The latter group was made up of healthy children of counsellors and physicians who were not campers. These two groups were included to provide a composite picture of the urinary coproporphyrin excretion in all age groups. The urinary coproporphyrin was determined by the method of Schwartz *et al.*(14). The urines were collected in colorless bottles containing 5 g of sodium carbonate and they were stored at 4°C. With this method of storage, the coproporphyrin content remained unchanged as long as 3 months after the collection and initial determination. Duplicate 5 ml aliquots were taken from the 24-hour urine samples and used for determination. The coproporphyrin in the test samples was determined fluorimetrically with a Calectron fluorimeter† employing a photomultiplier tube and with the Corning No. 5543 as the primary filter and Corning No. 2418 as the secondary filter. Values were determined from a standard curve obtained from a series of coproporphyrin solutions of known concentration.‡

Results. The urinary coproporphyrin excretion in children on the basis of age is shown in Fig. 1. Similar data plotted on the basis of body weight are shown in Fig. 2, and for surface area in Fig. 3.

The data are divided into groups and sum-

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* Aided by grants from the M&R Dietetic Laboratories and the Playtex Park Research Institute.

† Manufactured by Calectron, Inc., St. Paul, Minn.

‡ Kindly supplied by Samuel S. Schwartz, M.D.

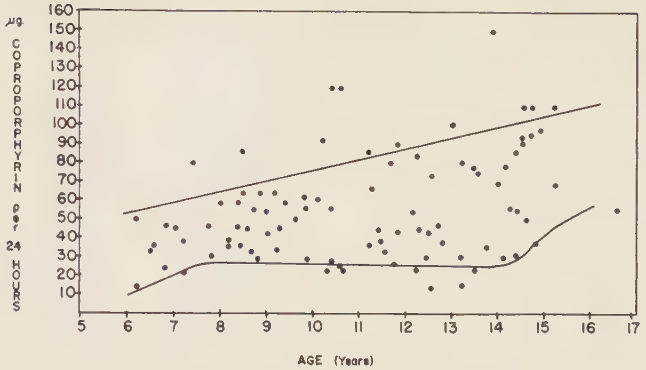

FIG. 1. Urinary coproporphyrin excretion in normal boys based upon age. The black line above represents one S.D. above the mean and the black line below represents one S.D. below the mean.

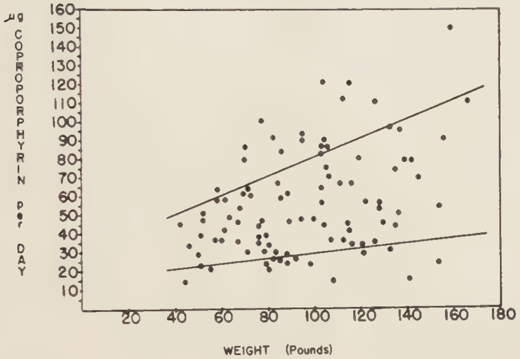

FIG. 2. Urinary coproporphyrin excretion in normal boys based upon body wt. The black line above represents one S.D. above the mean and the black line below represents one S.D. below the mean.

marized in Table I.

Discussion. From data on coproporphyrin excretion on successive days in a number of normal adults, Strait(17) has noted that there exists a simple relationship between the weight of the subject and the mean value of coproporphyrin excretion. He felt that the total urinary coproporphyrin excretion reflects primarily an endogenous metabolic function and that the coproporphyrin excretion fits more closely the relationship of body weight to blood volume than the relationship of body weight to surface area, the well known basal metabolic rate function. In studies with a small group of children, he found that their coproporphyrin excretion also depended on weight, but did not fit the extrapolated linear relationship described for the adults. Instead, the weight-coproporphyrin function for children may require an exponent of the weight somewhat greater than unity for the extrapolated fit.

Although the urine collections were not made over a number of days in each indi-

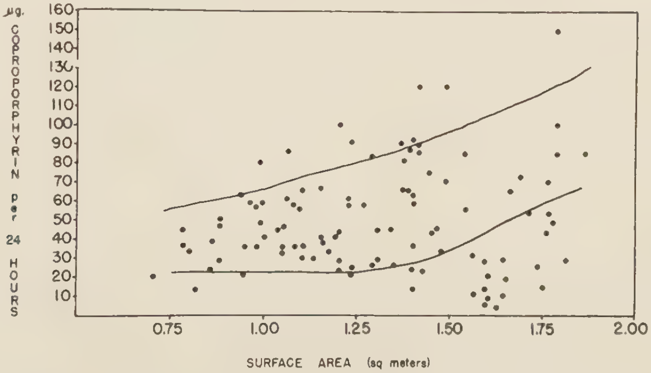

FIG. 3. Urinary coproporphyrin excretion in normal boys based upon body surface. The black line above represents one S.D. above the mean and the black line below represents one S.D. below the mean.

TABLE I (A). Mean Coproporphyrin Excretion of Boys 6 to 16 Years Based upon Age.

Years	No.	Mean copro. excr., µg/24 hr	S.D.
<6 yr*	4	41.7	14.9
6 to 7 " 11 mo	14	38.4	16.5
8 " 9 " 11 "	24	50.6	18.6
10 " 11 " 11 "	20	56.3	30.2
12 " 13 " 11 "	20	56.2	31.5
14 " 15 " 11 "	16	75.2	27.0
Adults*	9	145.5	63.6

* The under 6 years and adult group are included to show composite picture of coproporphyrin excretion at all ages.

TABLE I (B). Mean Coproporphyrin Excretion of Boys Aged 6 to 16 Based upon Body Weight.

Wt, lb	No.	Mean copro. excr., µg/24 hr	S.D.
41- 60	12	38.5	17.9
61- 80	22	47.4	19.5
81-100	17	48.5	23.6
101-120	22	62.8	29.4
121-170	21	66.2	32.8

TABLE I (C). Mean Coproporphyrin Excretion of Boys Aged 6 to 16 Based upon Body Surface Area.

Body surface, sq. meter	No.	Mean copro. excr., µg/24 hr	S.D.
.75-1.0	16	41.8	16.1
1.01-1.25	25	26.4	16.1
1.26-1.50	28	61.1	29.1
1.51-1.75	17	54.4	26.4
1.76-2.0	8	92.4	28.6

vidual as recommended by Strait, the data in general appear to confirm his concepts. Examining first the changes of coproporphyrin excretion on the basis of age, one is impressed by the moderate rise prior to the age of 6 and 7, the levelling off during the next 6 years, and the rapid rise during puberty and adolescence. This closely parallels the changes in body weight in normal boys and is confirmed by the steady rise of coproporphyrin when correlated with body weight as shown in Table I(B). Surface area did not appear to correlate as satisfactorily as weight. It would appear on the basis of these data that the best criterion for defining abnormal urinary coproporphyrin excretion in children would be in cases where the 24-hour collection is twice the standard deviation above the mean for the group the individual belongs to by weight.

Summary. 1. The urinary coproporphyrin excretion on 24-hour urine collections has been determined on a group of normal healthy boys aged 6 to 16 by the new improved method. 2. The urinary coproporphyrin level correlates closer with body weight than with age or surface area. 3. The urine coproporphyrin excretion in children is probably abnormal if the determination in a 24-hour collection is twice the standard deviation above the mean for the group the individual belongs to by weight.

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1. Watson, C. J., and Larson, E. A., *Phys. Rev.*, 1947, v27, 478.

2. Dunsky, J., Freeman, S., and Gibson, S., *Am. J. Dis. Ch.*, 1947, v74, 305.

3. Dobriner, K., Strain, W. H., Guild, H., and Localio, S. A., *J. Clin. Invest.*, 1938, v17, 761.

4. Rimington, C., *Nature*, London, 1937, v140, 105.

5. Watson, C. J., Hawkinson, V., Capps, R. B., and Rappaport, E. M., *J. Clin. Invest.*, 1949, v28, 621.

6. Watson, C. J., Sutherland, D., and Hawkinson, V., *J. Lab. and Clin. Med.*, 1951, v37, 8.

7. Sutherland, D. A., and Bollier, M., *Gastroenterology*, 1952, v20, 39.

8. Watson, C. J., *J. Clin. Invest.*, 1936, v15, 332.

9. Schmid, R., Hanson, B., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 459.

10. Schwartz, S., Keprios, M., and Schmid, R., *ibid.*, 1952, v79, 463.

11. Watson, C. J., Schulze, W., Hawkinson, V., and Baker, A. B., *ibid.*, 1947, v64, 73.

12. Watson, C. J., *J. Lab. and Clin. Med.*, 1951, v37, 29.

13. ———, *The relation of porphyrins to erythropoiesis.* New York: Science Press Printing Co., 1940.

14. Schwartz, S., Zieve, L., and Watson, C. J., *J. Lab. and Clin. Med.*, 1951, v37, 843.

15. Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, v168, 133.

16. Zieve, L., Hill, E., Schwartz, S., and Watson, C. J., *J. Lab. and Clin. Med.*, 1953, v41, 663.

17. Strait, L. A., Bierman, H. R., Eddy, B., Hrenoff, M., and Eiler, J. J., *J. Appl. Physiol.*, 1952, v9, 699.

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Growth Inhibition of a Biotinless Mutant of *Escherichia coli* by Purines and Pyrimidines.* (20795)

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It is well known that the biotin requirement of certain microorganisms can partially be replaced by aspartic acid(1), oleic acid(2), or a combination of these two substances (3,4). For *Lactobacillus arabinosus*, oxalacetate effectively replaces aspartate but other members of the Krebs cycle do not(5). For the "biotinless" mutant, *Escherichia coli* 58, which is an aerobic organism, a variety of four carbon dicarboxylic acids are effective in partially replacing the requirement for biotin(6). These observations led to the conclusion that biotin is involved in the synthesis of 4 carbon dicarboxylic acids from pyruvate and carbon dioxide(5,7,8). It has been postulated that biotin also plays a role in purine synthesis since biotin-deficient rats fix less $C^{14}O_2$ into their tissue guanine than do normal rats(9). When experiments were conducted to determine the biotin-sparing action of purines and pyrimidines for *E. coli* 58 it was found that these substances were inhibitory. In the presence of biotin, however, the purines and pyrimidines did not inhibit growth of the mutant and they were likewise innocuous toward the parent wild type *E. coli*, K 12.

Experimental. Escherichia coli 58,[†] (Gray and Tatum(10)) was carried on nutrient agar slants to which biotin was added (0.3 mγ per 10 ml slant). The slants were incubated at 37°C for 20-24 hours. The basal experimental medium(11) contained K_2HPO_4 7 g, KH_2PO_4 2 g, $(NH_4)_2SO_4$ 1 g, Na citrate 0.5 g, and $MgSO_4$ 0.1 g per liter. Growth experiments were conducted in Evelyn tubes. One ml of sterile 1% glucose was added to each tube containing 9 ml of the autoclaved basal medium. All additions were made at the expense of the basal medium. The tubes were inoculated with two drops of a cell suspension (adjusted to give a galvanometer reading of 91-93 with the Evelyn colorimeter, filter 620), made from 20-24-hour slants. Incubation was at 37°C without shaking. The *amount of growth* was measured in the Evelyn colorimeter using a 620 filter and setting the uninoculated tube at 100. Readings were taken after 20-24 hours incubation except in the growth curve experiments where readings were made at several intervals from 10-144 hours. The results are expressed as per cent of maximum growth calculated from density values. The positive control containing the basal medium and biotin was taken as 100%. The results reported represent averages of duplicate tubes. All experiments were repeatedly confirmed. The average per cent transmission value of the negative control (basal medium) was 95, of the positive control (basal medium + biotin) was 70, and that of the basal medium + aspartic acid was 82. The purine and pyrimidine compounds were obtained from the following companies: adenylic acid a and b, adenosine, adenine, thymine, guanine, and xanthine from Schwarz Laboratories; adenosine-5-phosphate, Bischoff; cytosine, Dougherty Chemicals; uracil, Eastman Kodak Co.; hypoxanthine, Nutritional Biochemicals Co. The free bases and nucleosides were autoclaved with the medium; nucleotides were sterilized by filtration and added to the tubes just before inoculation.

Results. The biotin requirement of *E. coli* 58 can be partially replaced by aspartic acid, the 4-carbon dicarboxylic acids, α-ketoglutarate or citrate(6). When attempts were made to improve growth of this organism in the absence of biotin by further supplementing the medium with other metabolites, it was found that purines and pyrimidines were inhibitory in biotin-free media but not in the presence of biotin.

As shown in Fig. 1, very low concentrations

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[†] Kindly supplied by Dr. J. Lederberg of the Genetics Department.

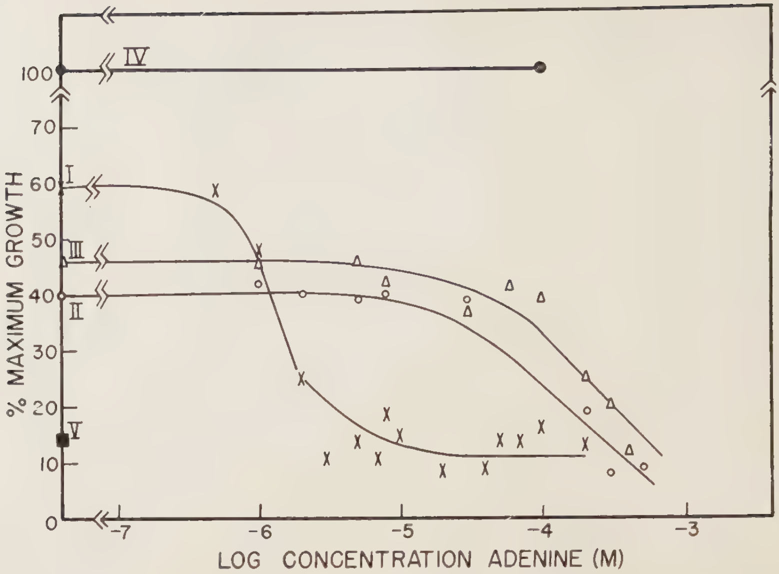

FIG. 1. Effect of adenine on the growth of *E. coli* 58.

The tubes contained:

I. Basal medium + 1 mg/ml DL-aspartic acid.
II. + " " succinic acid.
III. + " " α-ketoglutaric acid.
IV. + 0.2 mγ/ml biotin.
V. Basal medium.

of adenine (10^{-5} M), added to media containing aspartate, depressed growth to the level achieved on the basal medium. Some variation in the amount of inhibition at higher levels of adenine was observed. When growth was supported by supplementing the basal medium with succinate or α-ketoglutarate, the biotinless mutant was less sensitive to adenine although higher concentrations of the latter were inhibitory. Adenine did not inhibit growth when biotin was supplied (IV, Fig. 1). In other experiments, adenosine, adenosine-3′-phosphate (yeast adenylic acid isomer b), adenosine-5′-phosphate and adenosinetriphosphate inhibited growth of the biotinless mutant to about the same extent as an equimolar quantity of adenine. Nearly complete inhibition was obtained with 6 x 10^{-4} M concentrations of the nucleotides. With the exception of adenosinetriphosphate, these compounds were more strongly inhibitory in aspartate-containing media than in media containing succinate, malate or α-ketoglutarate. Adenosinetriphosphate inhibited growth even when succinate or α-ketoglutarate were added to replace, partially, the biotin requirement. Biotin overcame the inhibitory effect of adenosine and its derivatives for *E. coli* 58. These compounds did not depress the growth of *E. coli* K 12. Adenosine-2′-phosphate (yeast adenylic acid isomer a) did not inhibit growth of the biotinless mutant, but did not reverse the inhibition by adenine even when added at concentrations 10 times higher than adenine.

The effect of hypoxanthine is shown in Fig. 2. Inhibition was pronounced in media in which aspartate partially replaced the biotin requirement. When this partial requirement was met by succinate (II, Fig. 2) or α-ketoglutarate (III, Fig. 2), inhibition by hypoxanthine was negligible.

Fig. 3 presents the effect of uracil, cytosine, thymine, and xanthine on the growth of *E. coli* 58. A concentration of 10^{-6} to 10^{-5} M of either uracil or cytosine brought growth down to the level attained on the unsupplemented basal medium. Higher amounts of thymine (10^{-4} M) had to be added to cause significant inhibition and the concentration of

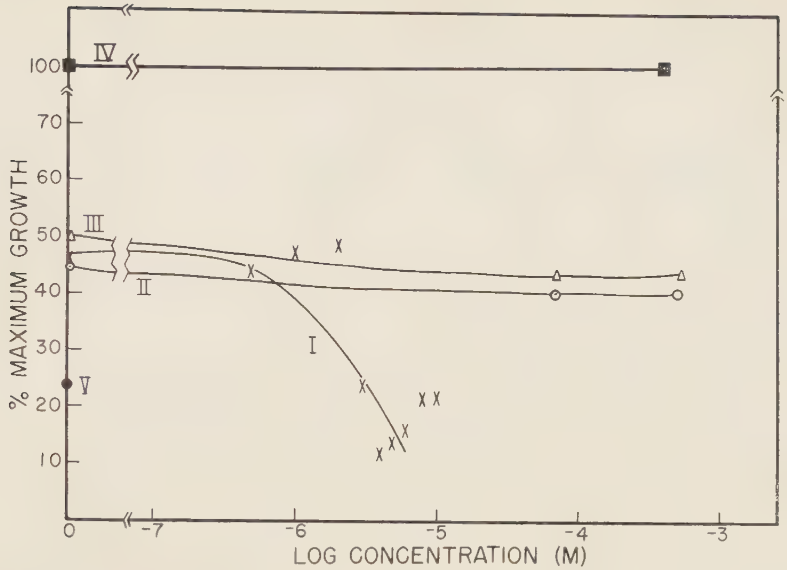

FIG. 2. Effect of hypoxanthine on the growth of *E. coli* 58.

The tubes contained:

I. Basal medium + 1 mg/ml DL-aspartic acid + hypoxanthine.
II. + 1 " succinic acid + hypoxanthine.
III. + 1 " α-ketoglutaric acid + hypoxanthine.
IV. + 0.2 mγ/ml biotin.
V. Basal medium.

xanthine had to be raised to 10^{-3} M before a similar effect was obtained. The data presented in Fig. 3 are from experiments in which the biotin requirements of the organism were partially replaced by aspartic acid. In two experiments in which the biotin requirements were partially replaced by succinic or α-ketoglutaric acid, uracil, cytosine, thymine, and xanthine at concentrations of 10^{-6} to 10^{-4} M depressed growth only slightly.

Additions of guanine failed to inhibit growth of *E. coli* 58 in the various media; however, owing to the great insolubility of guanine at a neutral pH, this substance could not be compared on an equimolar basis with the other inhibitors. Combinations of guanine and various pyrimidines did not reverse the inhibiting effect of adenine. None of the purine or pyrimidine bases inhibited growth of the wild type *E. coli*, K 12, when grown on either the basal medium or with supplements of aspartate or succinate.

Rates of growth of *E. coli* 58 up to 144 hours incubation are shown in Fig. 4. Both the rate of growth and the total growth finally achieved in media containing aspartate were depressed by adenine. The addition of biotin reversed both effects.

It has been consistently observed that in the basal medium and in the basal + aspartic acid, a secondary rapid growth phase takes place after the stationary phase has been reached. The suggestion that the mutant is able to synthesize some biotin after prolonged incubation was confirmed by assay((6) *Lactobacillus arabinosus*) of the cells grown in biotin-free media. Subcultures from these tubes, however, retain their biotin requirement.

Discussion. There are several plausible explanations of the inhibitory effect of purines and pyrimidines on the biotinless mutant of *E. coli*, but no logical explanation can be offered for the greater inhibition by these compounds in the presence of aspartate as compared with succinate or α-ketoglutarate. It is possible that the purines and pyrimidines inhibit by competitive interference with normal intermediary metabolites, for example, in the elaboration of the nucleotide units(12,13)

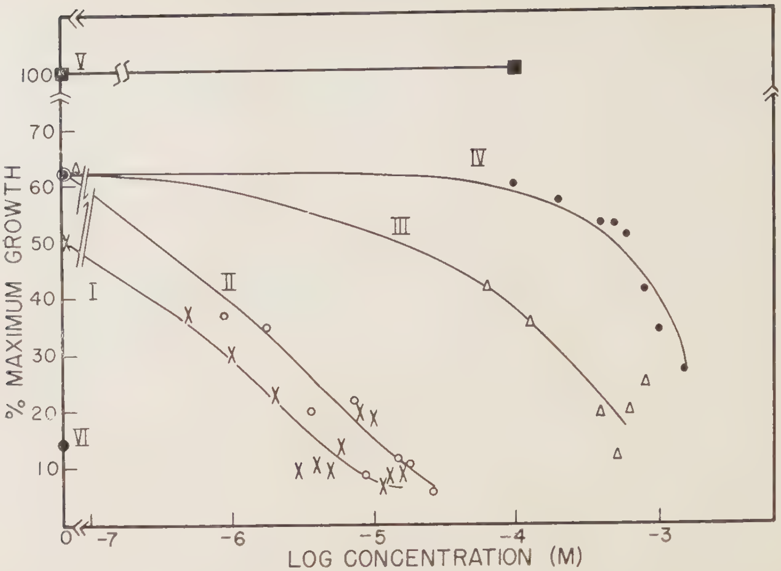

FIG. 3. Effect of uracil, cytosine, thymine and xanthine on growth of *E. coli* 58.
The tubes contained:
I. Basal medium + 1 mg/ml DL-aspartic acid + uracil.
II. + cytosine.
III. + thymine.
IV. + xanthine.
V. + 0.2 mγ/ml biotin
(plus any of the above purines or pyrimidines).
VI. Basal medium.

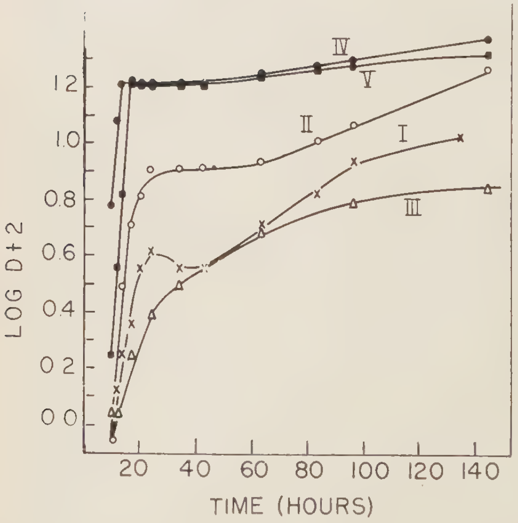

FIG. 4.
Inhibition of growth of *E. coli* 58 by adenine.
The tubes contained:
I. Basal medium.
II. Basal medium + 1 mg/ml DL-aspartic acid.
III. Basal medium + 1 mg/ml DL-aspartic acid + 10^{-4} M adenine.
IV. Basal medium + 1 mg/ml DL-aspartic acid + 10^{-4} adenine + 0.2 mγ/ml biotin.
V. Basal medium + 0.2 mγ/ml biotin.

which participate in nucleic acid synthesis. Pierce and Loring(14) have reported that adenosine inhibits growth of a pyrimidine-deficient mutant of *Neurospora* and that uridine and cytidine reversed the inhibitory effect. In the present work no such reversal could be obtained.

An inhibitory effect of adenine, guanine, and uracil on the growth of *Streptococcus faecalis* in the absence of serine was reported by Holland and Meinke(15). The inhibition could also be reversed by adequate levels of folic acid and in this respect is somewhat analogous to the one reported here.

Summary. The growth of the biotinless mutant, *Escherichia coli* 58, in a medium containing aspartic acid to replace partially the biotin requirement, was inhibited by adenine, adenosine, adenosine-3′- or 5′-phosphate, ATP, uracil, cytosine, hypoxanthine, and, to a lesser extent, by thymine and xanthine. Adenosine-2′-phosphate was not inhibitory. None of these compounds was inhibitory when biotin was added to the growth medium of strain 58, nor were they inhibitory to the parent strain *E. coli* K 12 which does not require biotin. When succinate or α-ketoglutarate, instead of aspartate, were added to the basal medium, adenine and its derivatives were less inhibitory and the other purines and pyrimidines did not significantly inhibit the growth of the biotinless mutant.

1. Stokes, J. L., Larsen, A., and Gunness, M., *J. Biol. Chem.*, 1947, v167, 613; *J. Bact.*, 1947, v54, 219.

2. Williams, V. R., and Fieger, E. A., *Ind. Eng. Chem. Anal. Ed.*, 1945, v17, 127.

3. Williams, W. L., Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, 1947, v170, 619.

4. Potter, R. L., and Elvehjem, C. A., *ibid.*, 1948, v172, 531.

5. Lardy, H. A., Potter, R. L., and Elvehjem, C. A., *ibid.*, 1947, v169, 451.

6. Armstrong, K. L., Thesis, University of Wisconsin, 1949.

7. Shive, W., and Rogers, L. L., *J. Biol. Chem.*, 1947, v169, 453.

8. Lardy, H. A., Potter, R. L., and Burris, R. H., *J. Biol. Chem.*, 1949, v179, 721.

9. MacLeod, P. R., and Lardy, H. A., *ibid.*, 1949, v179, 733.

10. Gray, C. H., and Tatum, E. L., *Proc. Nat. Acad. Sci.*, 1944, v30, 404.

11. Lederberg, J., *Methods in Med. Research*, 1950, v3, 5.

12. Greenberg, G. R., *J. Biol. Chem.*, 1951, v190, 611; *J. Am. Chem. Soc.*, 1952, v74, 6307.

13. Williams, W. J., and Buchanan, J. M., *J. Biol. Chem.*, 1953, v202, 253.

14. Pierce, J. G., and Loring, H. S., *ibid.*, 1948, v176, 1131.

15. Holland, B. R., and Meinke, W. W., *ibid.*, 1949, v178, 7.

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Effects of Growth Hormone upon Erythropoiesis in the Hypophysectomized Rat.* (20796)

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Chronic treatment with the growth principle fails to prevent(1-5) or overcome(6) the anemia characteristic of the hypophysectomized rat. Despite this lack of influence upon the peripheral blood values, two of the above groups of investigators(1,6) have found that somatotrophin (STH) induces considerable repair of the hypoplastic marrow of the hypophysectomized rat. The present research was undertaken to determine, in more detail, the influence of STH upon erythropoiesis and to uncover the cause for the discrepancy observed between the behavior of the peripheral blood and bone marrow elements.

Materials and methods. Twelve male rats of a modified Long-Evans strain, hypophysectomized 2 months previously and weighing 150-200 g at the beginning of the experiment, were employed. They were fed a complete laboratory ration and kept at temperatures of 70-80°F. Six of the rats received daily subcutaneous injections, for 7 days, of one mg of the Raben-Westermeyer(7) STH preparation‡ dissolved in 0.2 cc water acidified with 0.1 N HCl to pH 3.0. The 6 control hypophysectomized animals were injected with 0.2 cc acid water for the same period of time. Hematologic analyses were conducted shortly before and at the termination of the 7-day period. Reticulocyte counts were made from wet mounts stained with brilliant cresyl blue. One thousand cells were counted for each determination. Quadruplicate hematocrit values for heparinized blood, spun at 7000 RPM for 15 minutes, were obtained by a capillary micromethod. At the termination of the experiment, the rats were anesthetized with ether and exsanguinated, as fully as possible, by cardiac puncture. The volume of blood withdrawn from each animal was recorded. The femora and tibiae were dissected, cracked open and the bone marrow removed and pooled. The tissue was prepared in suspension form in homologous serum by drawing it up and down gently with a pipette. Smears were made and treated by Ralph's technic(8) using Harris' hematoxylin as a counterstain. With this method, the cytoplasm in both the mature red cells and the nucleated erythrocytes, in various stages of development, appears distinctly golden in color because of the specific staining of hemoglobin. The use of Harris' hematoxylin, as a nuclear stain, serves further to distinguish between the nucleated erythrocytes and the other marrow elements. Giemsa-stained smears were also prepared. At least 500 cells were counted for each animal to determine the percentages of nucleated erythrocytes. The humeri were fixed in Helly's fluid, decalcified by a formic acid-ion exchange resin preparation (Win-3000), sectioned in paraffin at 10 μ and stained with hematoxylin and eosin.

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‡ We are indebted to Dr. William Kleinberg, Princeton Laboratories Inc., Princeton, N. J., for the generous supplies of STH(G25-H) furnished.

TABLE I. Effects of Growth Hormone upon Erythropoiesis and Organ Weights of Hypophysectomized Rats (Means ± Standard Errors).

	STH	Acid water	P*
Change in wt (g)	+21.3± 1.43	—4.8± 1.3	<.001
Reticul. (%)	3.8± .81	1.0± .14	.001
Marrow nuc. RBC (%)	18.4± 2.61	9.2± 1.1	.007
Change in hematoc. (%)	—4.2± 1.1	—1.7± 1.3	.179
Vol. "drawn blood" (ml)	5.0± .91	3.2± .52	.083
Thyroid wt (mg)†	8.7± .73	6.8± .64	.062
Adrenal " "	11.7± 1.62	9.4± .35	.244
Seminal vesic. wt (mg)	45.1± 1.76	44.9± 3.63	>.80
Thymus wt (mg)	234 ±27.7	156 ±37.1	.083
Spleen wt (mg)	688 ±61.4	409 ±29.2	.002

* P is calculated from the distribution of Fisher's t. Values less than 0.05 have been considered significant.

† One of the 6 animals treated with STH and included in the table may have been partially hypophysectomized. It possessed a larger body wt and, at the time of sacrifice, its thyroid and adrenals weighed 11 and 19 mg respectively. The remainder of its values, however, fell within the range of the other 5 STH-injected rats.

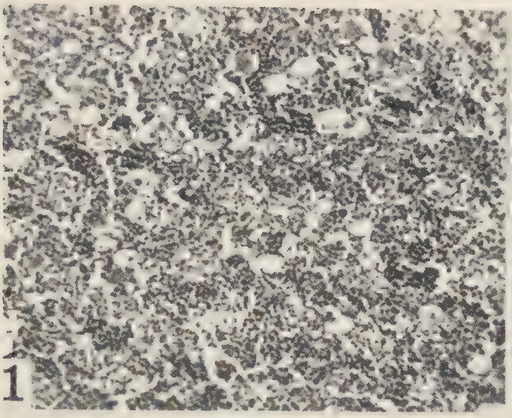
Results. Evidence is presented in Table I that STH stimulates erythropoiesis in the hypophysectomized rat. Thus significant reticulocytosis and increases in the percentages of nucleated marrow erythroid elements are apparent after a week's treatment with this agent. The marked reparative influence of STH upon the bone marrow structure of the relatively long-term hypophysectomized rat is evident from Fig. 1-4, inclusive. Despite these significant effects upon the marrow, the hematocrit values remain at the low level seen in untreated or in the acid water-injected controls.

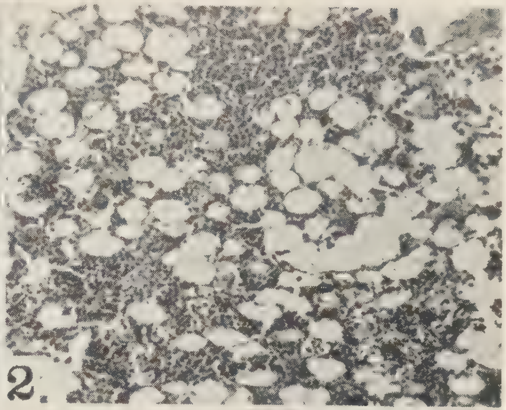
In Table I are also indicated the significant body weight gains displayed by the animals treated with STH. It is of interest that the Raben-Westermeyer preparation, in the dosage given, does not affect significantly the weights of the thyroid, adrenals, seminal vesicles and thymus. Splenic weights, however, are elevated with this treatment.

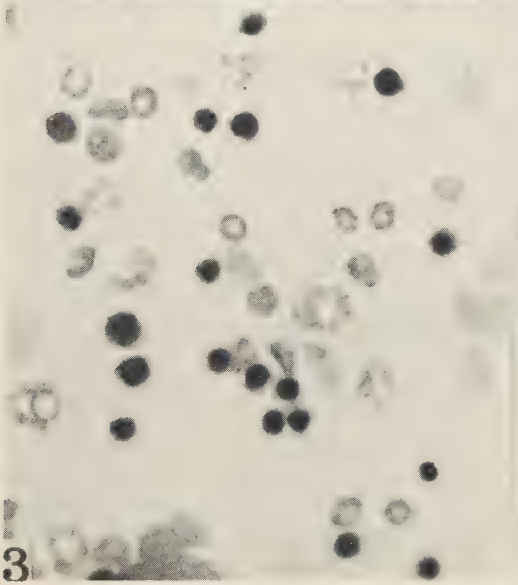
At the termination of the experiment, it was found that consistently greater volumes of blood could be aspirated by the same investigator (GJF) from the rats injected with STH than from those given the acid water. The difference in mean values, however, is just outside the selected $P < 0.05$ limits of significance ($P = 0.08$).

Discussion. The present experiments indicate that the Raben-Westermeyer preparation of STH, possessing little or no gonadotrophic, corticotrophic or thyrotrophic hormone activity in the dosages used, exerts a significant erythropoietic effect in the long-term hypophysectomized rat. It remains to be determined whether this influence is a specific one imparted directly to the marrow or is merely a reflection of the capacity of STH to augment the food intake, thereby increasing the nutritional status of the animal.

The apparent lack of response to STH of the peripheral red cell numbers, hematocrit and hemoglobin values, as noted by others and in this report, is probably a reflection of







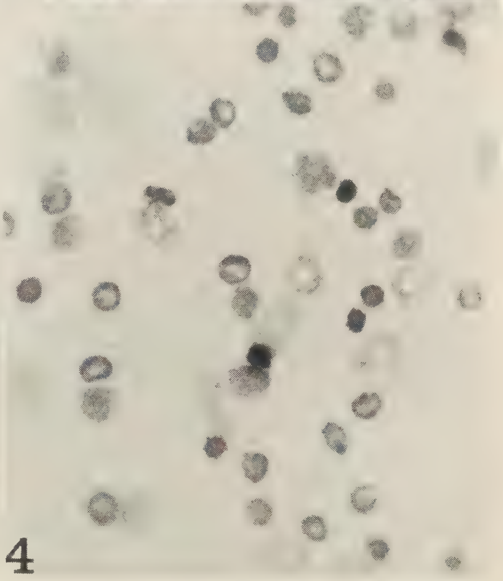


FIG. 1. Humeral bone marrow section of hypophysectomized rat treated daily for 7 days with 1 mg STH dissolved in 0.2 cc acid water. Considerable repair of post-hypophysectomy hypoplasia is observed (cf Fig. 2). Helly fixation, hematoxylin-eosin. ×140.

FIG. 2. Humeral bone marrow section of hypophysectomized rat treated daily for 7 days with 0.2 cc acid water. No effect is apparent upon the typical post-hypophysectomy hypoplasia. Helly fixation, hematoxylin-eosin. ×140.

FIG. 3. Bone marrow smear from hypophysectomized rat treated daily for 7 days with 1 mg STH dissolved in 0.2 cc acid water. Note greater concentration of nucleated erythrocytes (darkly stained) compared to Fig. 4. Benzidine, Harris' hematoxylin preparation. ×695.

FIG. 4. Bone marrow smear from hypophysectomized rat treated daily for 7 days with 0.2 cc acid water. Concentration of nucleated erythrocytes is considerably less than that observed in Fig. 3. Benzidine, Harris' hematoxylin preparation. ×695.

the ability of this hormone to augment the plasma volume, a phenomenon observed strikingly in the hypophysectomized dog(9). It appears to be of sufficient intensity in the rat to obscure the rise in peripheral red cell values that might be anticipated from the observed erythropoietic action of the hormone. In view of the strong reticulocytosis evoked by STH, it is unlikely that it acts by inhibiting the release of erythrocytes from the marrow.

Support for the contention that an increase in plasma volume has occurred in the rats receiving STH is derived from the present observations that larger quantities of blood are obtainable from the hearts of these animals at the time of sacrifice. In this connection, the experiments of Lippman(10) have indicated that "drawn blood" values are a reflection of the actual blood volume levels. Also in line with the concept of a plasma volume change is the finding that a significant rise occurs in red cell counts and hemoglobin values within a few days following cessation of STH treatment in the hypophysectomized rat(6).

Summary. Administration of STH to hypophysectomized rats results in peripheral reticulocytosis and increases in the percentages of nucleated erythroid elements within the bone marrow. Considerable repair of the hypoplastic marrow is induced by this agent. A rise in peripheral erythrocytic values fails to occur probably because of a concomitant increase in plasma volume.

1. Meyer, O. O., Stewart, G. E., Thewlis, E. W., and Rusch, H. P., *Folia Haematol.*, 1937, v57, 99.
2. Querido, A., and Overbeek, G. A., *Arch. Internat. de Pharmacodyn. et de Thérap.*, 1938, v59, 370.
3. Gaebler, O. H., and Mathies, J. C., *Endocrinology*, 1951, v48, 623.
4. Van Dyke, D. C., Simpson, M. E., Garcia, J. F., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 574.
5. Crafts, R. C., *Endocrinology*, 1953, v53, 235.
6. Gerstner, R., and Gordon, A. S., unpublished.
7. Raben, M. S., and Westermeyer, V. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 550.
8. Ralph, P. H., *Stain Technol.*, 1941, v16, 105.
9. Campbell, J., Hausler, H. R., Munroe, J. S., and Davidson, I. W. F., *Endocrinology*, 1953, v53, 134.
10. Lippman, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 188.

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Infrared Spectroscopy of Liver Glycogen in Normal and Irradiated Adult and Fetal Rats.* (20797)

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Previous studies(1) on rabbits demonstrated infrared absorption spectra of liver for the region 1-15 μ. Two types of absorption bands were found within 2 different intervals of that region: 1. Bands below 8.1 μ, which were very similar to those of the common "protein polyamide" vibrations(2) and occur not only in liver but in all other tissues (3.04 μ NH stretching, 3.4 μ CH stretching, 6.0 μ CO stretching, the deformation vibration of NH at 6.44 μ, the deformation vibration of CH at 6.84 μ, the CH 3 vibration at 7.26 μ, and an unassigned band at 8.1 μ). 2. Bands between 8.1 μ and 11 μ which have been shown to be characteristic for liver tissue, "fingerprint bands" (double bands at 9.70-9.64 μ and 9.23 μ). It was also noted that after 2-3 days starvation followed by insulin shock, one of the characteristic double bands at 9.70-9.64 μ disappeared, while the other at 9.23 μ remained, although greatly reduced in strength.

The present paper reports differences in infrared spectroscopic findings in the liver of adult and fetal rats, definite assignment of one characteristic band to glycogen, and spectroscopic studies of the effect of ionizing irradiation on liver glycogen in adult and fetal rats.

Materials and methods. Female albino rats of about 200 g were used for all experiments. Fetal livers were obtained in the 2nd-3rd week of development. Animals were kept for about one week on a balanced diet *ad libitum*, and then examined either non-fasted, fasted for 24 hours, or after feeding various sugars by stomach tube 4 hours preceding the experiment. When tube feeding was employed the tube was passed under light nembutal anesthesia on both experimental and control animals. All experiments were timed to take place at approximately 1 P.M. The animals were killed by decapitation and the livers quickly removed and frozen. For spectroscopy, frozen sections of 25-50 μ thickness were prepared, placed upon silver chloride plates and dried at low temperature. Histologic studies ruled out the possibility of complicating disease. The *irradiation* experiments comprised administering a single dose of total body irradiation of 600-19000 r to non-pregnant animals and of 150-400 r to pregnant animals. The technic of x-ray irradiation has been described elsewhere(3). Adult non-pregnant animals were sacrificed one or 2 days after the irradiation, fetuses 24 hours after maternal irradiation. The method of *infrared spectroscopy* of frozen and dried tissue sections has been described(4). For semi-quantitative estimate of the substances producing the characteristic band of liver tissue, ratios of optical densities of this band and a "protein vibration," *e.g.*, the deformation vibration of NH at 6.44 μ, were determined. Since such ratios between optical densities of unknown to internal standard are independent of thickness of sections according to Beer's law, they can be used for estimations in sections of varying thicknesses.

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* This work was carried out under contract with the U. S. Atomic Energy Commission.

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Results of qualitative infrared spectroscopy of adult and fetal liver tissue. The upper part of Fig. 1 gives transmittancy curves which were obtained from sections of about the same thickness (50 μ) of adult and fetal liver from non-fasted rats and from films of commercial glycogen.

The spectra of both adult and fetal liver show 2 different types of absorption bands similar to those found in rabbits, *e.g.*, "protein polyamide" vibrations below 8.1 μ and strong characteristic double bands at 9.70 μ-9.60 μ, and 9.23 μ, and weaker bands at 8.65 μ. Still weaker bands at 10.30 μ and 10.70 μ were seen in most of the spectral recordings, but do not appear regularly in the illustrations. Close inspection of the transmittancy curves indicates distinct differences between spectra of adult and fetal liver. The most characteristic band of the tissue spectrum is located at somewhat lower wavelength in adult liver (9.60 μ) than in fetal liver (9.70 μ). The relative strength of those "specific bands" is always weaker than the strength of the "protein bands" at 6.44 μ in adult liver while it is almost equal or even stronger in fetal liver.

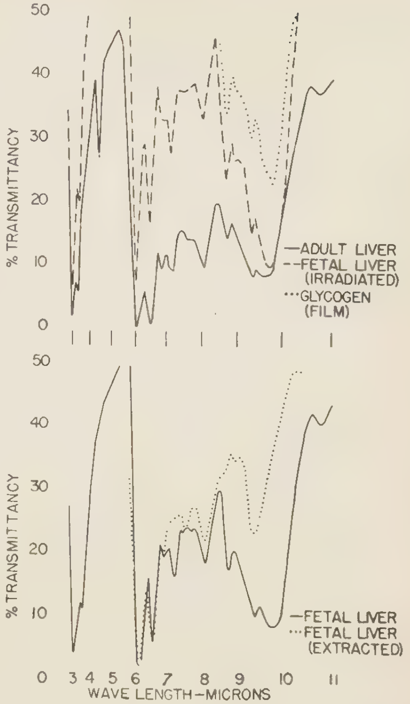

FIG. 1. Comparison of infrared absorption spectra of adult and fetal liver tissue and commercial glycogen (upper curves); effect of extraction with cold 10% trichloracetic acid on spectra of fetal liver (lower curves).

Assignment of characteristic bands of the liver spectrum. Glycogen is known to be contained in liver tissue in so high concentration that it seemed reasonable to investigate its role in producing the characteristic liver spectra. Comparison of the liver spectrum above 8.1 μ with the corresponding spectrum of commercial glycogen (upper part of Fig. 1) shows the great similarity of both spectra. Spectra of fetal liver and glycogen show identically located bands at 8.65 μ, 9.23 μ, and 9.70 μ. Spectra of adult liver show similar locations of bands with the exception of the characteristic double band which is found at somewhat lower wavelength (9.60 μ).

Extraction of mounted dried liver sections with either cold or hot chloroform, or cold or hot ethanol did not significantly affect the characteristic bands thus precluding any important contributions to the spectrum by lipids. Extraction of liver sections with cold 10% trichloracetic acid, on the other hand altered the spectra decisively. One of the characteristic double bands (9.70 μ-9.60 μ) was either completely absent or greatly reduced and the other at 9.23 μ although present, was much weaker after the extraction. The lower part of Fig. 1 demonstrates this effect of the extraction on a fetal liver section. The illustration, in addition, shows that the extraction, which so greatly changes the characteristic bands of liver tissue above 8.1 μ, does not affect the strength of the protein bands below 8.1 μ. Acid soluble phosphates which have weak absorption bands in the 9-10 μ region(5) are contained in liver in too small concentration to make any detectable contribution to the spectrum. It is, therefore, reasonable to assume that the trichloracetic soluble substance is glycogen, since only glycogen appears to have all 3 necessary qualities, *e.g.*, identical infrared spectra, sufficient tissue concentration, and solubility in cold trichloracetic acid. Additional proof for the assignment of the described infrared bands to glycogen has been obtained by comparing the liver spectra of adult fasted rats with liver spectra of animals given sugar after fasting and further comparsion of such spectra with glycogen (Fig. 2).

The liver spectrum of the fasted rat as a whole resembles that of liver extracted with cold trichloracetic acid, which has been considered to be practically glycogen-free. The liver spectrum of the sugar-fed rat, on the other hand showed the characteristic glycogen bands previously described except for slight variations in location of the strong specific band which was found at about 9.65 μ after sucrose and at 9.61 μ after glucose feeding. Contribution of glycogen to the liver spectrum of sugar-fed rats is even more suggested from difference curves which have been obtained by deduction of the absorption of "fasted liver" from the liver spectrum of the sugar-fed animals. The characteristic 8.1-11 μ region of such a difference curve (interrupted line) closely resembles a transmittancy curve of glycogen.

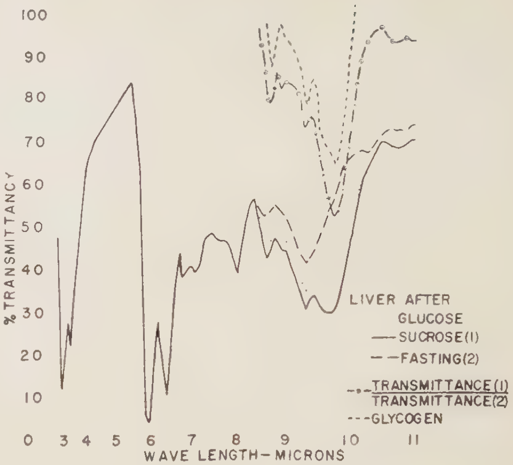

FIG. 2. Comparison of infrared spectra of liver of fasted and sugar fed litter mates and commercial glycogen.

The results of the assignment study strongly suggest that the characteristic strong band of normal liver (9.70 μ-9.60 μ) is produced by glycogen. The band at 9.23 μ is not caused by glycogen alone, but in part by vibrations of other substances. Almost complete disappearance of the 9.23 μ band after extraction of liver with hot trichloracetic acid indicates that it is produced in part by nucleic acids which have medium strong bands at that wavelength(6). The finding that the strongest band of glycogen in the 8.1-11 μ region is constantly observed at 9.70-9.60 μ in liver tissue is of practical importance. This "specific band" of the liver spectrum is distant enough from the "mixed band" at 9.23 μ that significant variations of its strength and location deserve careful consideration.

Semiquantitative estimation of liver glycogen. Ratios between optical densities of the glycogen bands at 9.70-9.60 μ and optical densities of the "protein bands" at 6.44 μ (designated as K values) have been used for semiquantitative estimation of the relative glycogen content of liver. With thickness of frozen sections kept at about 25 μ, it has been possible to obtain accurate measurements of bands and to calculate significant K values in dried rat liver sections with the exception

TABLE I. Quantitative Estimations in Liver Tissue.

Normal rats				Irradiated rats					
No. of rats		λ	K	No. of rats		Dose, r	Hr after exposure	λ	K
Adult				Adult					
Fasted	13	9.70	.279 ± .033*	Fasted	7	1800	48	9.63	.471 ± .019*
Non-fasted	27	9.60	.595 ± .019	Non-fasted	20	600–1800	48	9.60	.571 ± .027
Sucrose fed	9	9.65	.582 ± .021	"	8	19000	1½	9.60	.453 ± .015
Glucose fed	9	9.61	.449 ± .011						
Fetal				Fetal					
Fasted	5	9.67	.619 ± .065						
Non-fasted	24	9.70	1.002 ± .025	Non-fasted	20	250–400	24	9.71	.976 ± .062
Glucose fed	9	9.71	.853 ± .069						

λ = Wave length—μ; $K = \dfrac{\text{Optical density at 9.70–9.60 } \mu}{\text{Optical density at 6.44 } \mu}$

* Means ± S.E.

of those from fasted adult rats. In this instance, when K values were calculated for exactly 9.70 μ though no glycogen band was detectable, the values are not a measure of glycogen, but caused mainly by non-specific absorptions and energy losses from reflection and scattering. These K values of adult fasted rats are given only to show the order of magnitude and relative constancy of the unspecific factors involved in the semiquantitative glycogen estimations. No attempt at correction of K values for those factors was made since it is thought that such a correction would not improve the relative value of our estimations except in the case of very small glycogen contents. This spectroscopic method has been used for screening differences in liver glycogen of adult and fetal rats kept under various nutritional conditions as well as after total body x-ray irradiation. Significant results of these studies are summarized in Table I.

Liver glycogen in adult and fetal rats. The table demonstrates that K values of fetal liver are considerably higher than those of the adults, in the non-fasted state in excess of 68%, in the fasted state, over 120%. The spectroscopic differences are so great that they are many times apparent from simple inspection of the spectra. Maternal fasting of 24 hours duration, for example, distinctly reduces the K values in fetal liver, but enough glycogen has remained to produce a rather strong glycogen band. Fasting of same duration in the adult, on the other hand, reduces the liver glycogen to such low values, that the glycogen band disappears and K values calculated for 970 μ are mostly caused by the unspecific factors described previously. Difference of storage of liver glycogen in adult and fetal rats is also apparent from study of K values in sugar-fed animals. The relative amount of glycogen after sugar feeding is much higher in fetal than in adult liver.

Effect of ionizing irradiation on liver glycogen. As shown by the K values in Table I, x-ray irradiation produces both an "acute effect" and a "delayed effect" upon liver glycogen, dependent on the nutritional state of the animal and the time elapsed after the exposure. The "acute effect" which was observed in non-fasted rats 1½-3 hours after exposure to 19000 r is characterized by a moderate drop in liver glycogen. The "delayed effect" noted in fasted animals 48 hours after irradiation with 1800 r consists of a significant increase of liver glycogen. The "delayed effect" can be recognized even from comparison of infrared spectra of irradiated animal and fasted control (Fig. 3). As a result of these opposing effects, non-fasted animals do not show significant changes of liver glycogen up to 48 hours after exposure to moderately high doses of x-rays (adults, 600-1800 r; fetus, 250-400 r maternal irradiation) provided that diarrhea or loss of appetite do not influence food absorption (Table I).

Discussion. It is apparent from the data presented that qualitative and quantitative infrared spectroscopy of liver sections provide

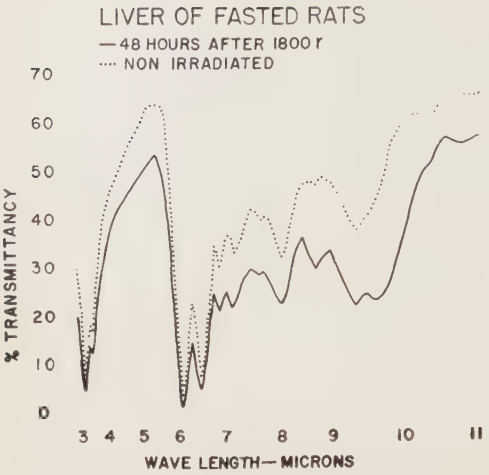

FIG. 3. Infrared absorption spectra of liver of non-irradiated and irradiated fasted rats.

an additional useful tool for study of liver glycogen. The variation of location of the "specific glycogen band" in adult and fetal liver is interesting when we consider that we did not find a single instance in which that band occurred exactly at 9.70 μ in normally fed adult animals, while it was regularly seen at that location in fetal liver and in commercial glycogen. Only in very few liver sections of adult animals fed with relatively high amounts of sucrose did we actually see that band close to 970 μ. This difference of location of the band exceeds by far the error of the method (±0.01 μ) and statistical analysis of data from all groups of non-fasted rats proved it to be statistically significant ($P < 0.01$). Two possible explanations for this finding which are still under investigation are offered. 1. The differences in wavelength may be related to different branching of the glycogen molecule. This possibility would fit into the findings of Cori *et al.*(7) who observed differences of end group figures in adult and fetal glycogen. 2. The variation of wavelength may be due to different protein binding of glycogen, with "protein bonded glycogen" producing vibrations at wavelengths somewhat different from those of "free glycogen." The assumption that glycogen rich liver contains mainly free glycogen would be in agreement with the concept of Willstaetter(8) who thought that "in glycogen rich liver the protein content was insufficient to bind a larger part of the glycogen." This explanation is further suggested by recent studies in another species(9) which show that almost all fetal glycogen can be extracted with cold trichloracetic acid while adult glycogen is not so easily extractable in this way.

The high glycogen content of fetal liver previously described and its response to maternal fasting and feeding are in agreement with studies of previous investigators who considered fetal glycogen more "mobile" with a tendency to fall or rise rapidly with fluctuations of maternal blood sugar(10).

Our results on the effect of ionizing irradiation upon liver glycogen tend to clarify seemingly conflicting reports that small doses (500 r) raise liver glycogen in fasted animals (11) and extremely high doses (110000 r), which actually killed the animal in 3½ hours, lower it in non-fasted animals(12). Our findings confirm and amplify both reports. An "acute effect" setting in 1½-3 hours after irradiation, is associated with loss of glycogen and a "delayed effect" occurring 48 hours after exposure, with an increase. The "acute effect" can obviously be recognized only in the non-fasted and the "delayed effect" can be observed best in the fasted animal.

Summary. 1. Frozen and dried sections of adult and fetal liver were studied by infrared spectroscopy. 2. The infrared absorption band found at 9.70-9.60 μ is definitely assigned to glycogen. 3. Differences of location of this band in adult and fetal liver are discussed. 4. A spectroscopic method for semiquantitative estimation of glycogen in liver sections is described. 5. Relative glycogen content in adult and fetal liver under various nutritional conditions is reported. 6. Studies of liver glycogen after x-ray irradiation show the existence of an "acute" and "delayed" effect, the former decreasing, and the latter increasing liver glycogen.

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1. Schwarz, H. P., Riggs, H. E., Glick, C., Cameron, W., Jaffe, B., and Trombetta, L., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 267.

2. Darmon, L. E., and Sutherland, G. B. B. M., *J. Am. Chem. Soc.*, 1947, v69, 207.

3. Schwarz, H. P., Riggs, H. E., Glick, C., McGrath, J., Cameron, W., Bayer, E., Jr., and Childs,

R., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 467.

4. Schwarz, H. P., *Applied Spectroscopy*, 1952, v6, No. 4.

5. Blout, E., and Fields, *J. Biol. Chem.*, 1949, v178, 334.

6. Daasch, L. W., and Smith, D. C., *Anal. Chem.*, 1951, v23, 853.

7. Illingworth, B., Larner, J., Jr., and Cori, G. T., *J. Biol. Chem.*, 1952, v199, 631.

8. Willstaetter, W., and Rhodewald, M., *Z. f. Physiol. Che.*, 1934, v224, 103.

9. Szepsenwol, J., and Partridge, M. H., *Am. J. Physiol.*, 1952, v168, 375.

10. Stuart, H. A., and Higgins, G. M., *ibid.*, 1935, v111, 590.

11. Levy, B., and Rugh, R., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 223.

12. Ross, M. H., and Ely, J. O., *J. Cell Comp. Physiol.*, 1951, v37, 163.

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An Hemostatic Defect Associated with Dextran Infusion. (20798)

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(Introduced by Victor M. Sborov.)

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Dextran, a glucose polymer of high molecular weight, has been developed as a plasma expander(1). During the course of studies at Walter Reed Army Hospital on the metabolic effects of dextran, 2 patients developed a bleeding tendency. One patient, after receiving 6000 ml of 6% dextran over a 6-day period developed repeated epistaxis and bled from a duodenal ulcer. The second patient, after receiving 14000 ml of 6% dextran over a period of 14 days, bled for more than 30 minutes from an incised sebaceous cyst. The bleeding time and prothrombin time were prolonged in both cases. Detailed studies were initiated to determine whether the administration of dextran provoked an hemostatic defect.

Methods. Six lots of commercially available dextran from 4 manufacturers were tested. Studies were done on normal young adult subjects who received intravenously varying amounts of 6% dextran. Bleeding time was measured following a stab incision with a Bard-Parker No. 11 blade through the dependent ear lobe. Prothrombin activity was measured by the one-stage Quick method; the clotting time was done in siliconized glassware. Plasma coagulation factors V and VII (labile and stable factors), prothrombin consumption, platelet count, clot retraction, antithrombin titer, and plasma dextran levels were determined(2,3). The subjects in these studies were divided into 3 groups.

Results. The first group of 11 patients received 1000 to 1500 ml of dextran daily until the bleeding time was prolonged to greater than 10 minutes. The total amount of dextran required to prolong the bleeding time varied from 1500 ml given in a single infusion to 6500 ml given over a period of 5 days. Determinations were carried out at least 4 times before each study and at 3-hour intervals for 9 to 12 hours after infusion. The bleeding time exceeded 12 minutes in 4 subjects and 30 minutes in 7 subjects. The prolonged bleeding times were usually not present immediately after infusion, but were maximal 3 to 9 hours after completion of the last infusion. With one exception the bleeding time returned to normal in 24 hours. Concomitant with the increased bleeding time, progressive diminution of prothrombin activity was noted with each repeated infusion of dextran in all subjects. The maximum depression of activity after infusion varied from 75-50% of normal. This depression of prothrombin activity was partially corrected by the addition of factors V and VII. Fig. 1 depicts the typical changes observed.

The clotting time was never abnormal, even when the bleeding time exceeded 30 minutes. Platelet counts were not below 150000/cu mm

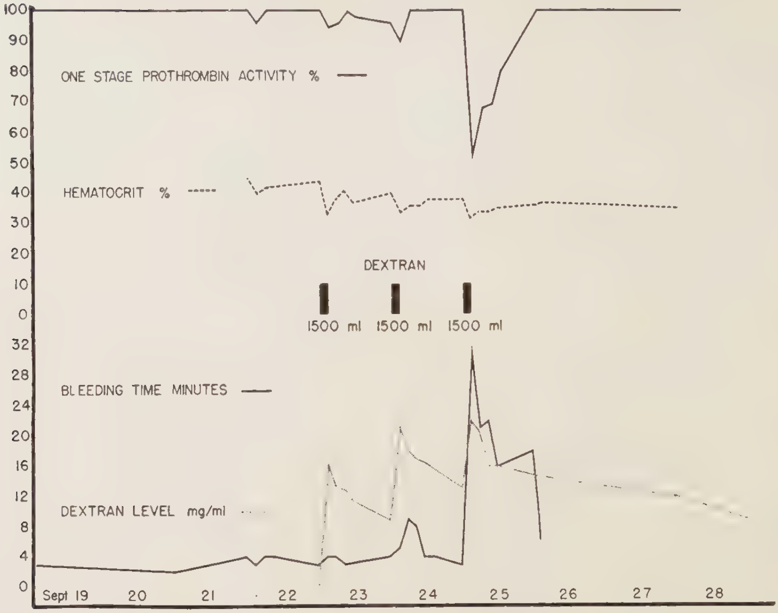

FIG. 1. Typical changes with dextran infusion observed in one subject.

(normal 200000 to 300000) in any patient. Prothrombin consumption was normal, and no circulating antithrombin was demonstrable. Clot retraction was normal. The fibrinogen level decreased from 400-500 mg % to 200-300 mg % at the end of the infusion; it returned to normal within 24 hours and was not significantly depressed when the bleeding time was maximal. Serial blood volume determinations using Cr^{51} tagged red blood cells(4) carried out on 3 patients receiving dextran daily showed that the maximum plasma volume expansion occurred immediately after the infusion and progressively declined thereafter.

After 1000 ml or more of dextran had been administered urinalyses showed the presence of renal epithelial cells, hyaline casts, and occasionally red cell casts and oval fat bodies in the sediment. The changes persisted throughout the period of administration and for 2-3 days thereafter. There was no proteinuria. The creatinine clearance and phenolsulfonphthalein excretion tests were normal throughout.

The second group of 15 subjects received a single infusion of 500 ml of 6% dextran. Bleeding time and prothrombin activity were determined before, immediately after, and at 3, 6, and 9 hours following infusion. Minimal depression of prothrombin activity to 85% of normal occurred in 3 of these subjects following infusion. Two of these subjects showed an increase in bleeding time from 3 and 4 minutes to 8 and 9 minutes respectively.

The third group of 50 subjects received a single infusion of 1000 ml of dextran. Bleeding times were determined before, immediately after, and at 3, 6, and 9 hours after infusion. Fourteen of the 50 developed bleeding times greater than 10 minutes within 9 hours following the infusion. Six hours following the infusion the bleeding time was greater than 30 minutes in one subject and greater than 3 hours in 2 other subjects. One of the latter had a bleeding time of 11 minutes 24 hours after the infusion. The remaining subjects had bleeding times well within the normal range 24 hours after the infusion. Table I summarizes the results in the 3 groups.

Two patients with chronic hepatitis were given 1000 ml of dextran daily for 10 days. Bleeding times and prothrombin times were done daily. The first of these patients showed a change of bleeding time from 3 to 24 minutes and a depression of prothrombin activity from 50-30% of normal. These values were

TABLE I. Relationship of Total Amount Dextran Infused to Prolonged Bleeding Time.

Cum dose of dextran	Days of infusion	Total No. subjects	Subjects with bleeding times >10′
500	1	15	0
1000	1	57	14
1500	1	8	2
2000	2	4	1
2500	2	4	2
3000	2	5	2
3500	3	2	1
4000	3	1	1
4500	4	2	1
5000	4	2	1
6500	5	1	1

prolonged for several days following the last dextran infusion. The second patient showed a change of bleeding time from 4-14 minutes and a depression of prothrombin activity from 95% to 35% of normal. In this patient the bleeding time was normal in 24 hours, while the prothrombin activity returned to normal in 4 days.

Discussion. It is generally accepted that the bleeding time is not usually prolonged unless the prothrombin activity is less than 10% of normal. Since the depression of prothrombin activity observed here is not of this magnitude, it is felt that the prothrombin deficiency cannot explain the increase in bleeding time. Because the maximal increase of plasma volume does not coincide with the prolongation of bleeding time, it would appear that the phenomenon is not correlated with intravascular volume changes. The defect may be related to abnormalities of hemostasis at the site of injury.

It should be emphasized that, excepting the patients with hepatitis, the present study has been carried out on normal subjects, in contrast to an army sponsored clinical trial of dextran in surgical patients and battle casualties, wherein some 4000 units of dextran were given to 2000 individuals with no report of hemorrhagic disease(5). In the present study the subjects were not under severe stress and were normovolemic prior to infusion. These differences in the recipients may prove important in determining the clinical significance of the phenomenon.

Summary. An hitherto undescribed hemostatic defect has been observed in normal subjects after infusion of dextran. This defect was characterized principally by a prolonged bleeding time. It was possible to produce this defect in all subjects in whom sufficient dextran was infused. The amount of 6% dextran required to produce this defect varied from 1000 to 6500 ml. Changes in plasma coagulation factors were minor and not sufficient to cause a prolonged bleeding time. The clinical significance of the hemostatic defect requires investigation.

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1. Gropper, A. L., Raisz, L. G., and Amspacher, W. H., *Surg., Gynec., and Obst.*, 1952, v95, 521.

2. Metcalf, W., and Rousselot, L. M., *J. Lab. and Clin. Med.*, 1952, v40, 901.

3. Scott, R., and Crosby, W. H., *Blood*, 1953, v8, in press.

4. Sterling, K., and Gray, S. J., *J. Clin. Invest.*, 1950, v29, 1604.

5. Medical Research and Development Board, Office of The Surgeon General, U. S. Army. Memorandum dated 7 May 53, "Clinical evaluation of dextran, final report".

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Antiviral Action of a Mold Filtrate on Experimental Poliomyelitis in Cynomolgus Monkeys.* (20799)

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Recent investigations have indicated that the crude filtrate of a penicillium mold, referred to as M-8450, has antiviral action against certain neurotropic viruses such as MM, Semliki Forest, and Type 2 poliomyelitis in mice(1,2) and against all 3 types of poliomyelitis in tissue culture(3). No activity was seen in mice against Lansing strain of poliomyelitis virus given intracerebrally, fixed rabies virus, or against a variety of non-neurotropic viruses(1). In view of the effectiveness of M-8450 against poliomyelitis in mice inoculated intraperitoneally and in tissue culture it was of interest to determine the effectiveness of this substance in monkeys. This report describes the effect of M-8450 in cynomolgus monkeys infected subcutaneously with Type 1 poliomyelitis virus.

Materials and methods. Young male and female cynomolgus monkeys, weighing between 1.2 and 2.5 kg, were used in this study. They were infected with approximately 10 PD_{50}'s of the Mahoney strain of Type 1 poliomyelitis virus and observed daily for the onset of paralysis for periods of at least 30 days. In previous tests(1,2) of the antiviral activity of M-8450 it was demonstrated that the mold filtrate was most effective in suppressing the experimental disease when both that virus and the filtrate were injected peripherally. Therefore, in tests with monkeys the Mahoney strain was employed because it is capable of inducing paralysis following subcutaneous inoculation. The M-8450[†] was obtained as the frozen-dried crude broth and stored at –70°C. It was rehydrated to original volume with sterile distilled water and administered intraperitoneally. In 3 of the 4 experiments, treated monkeys were given 100 ml of the rehydrated crude filtrate in 4 divided doses, 2 doses being given on the day before virus, the third 4 hours before, and the fourth 4 hours after virus inoculation. No signs of toxicity were observed in any of the mold-filtrate treated monkeys. In view of the large volumes of fluid involved, virus control monkeys in the first and third experiments were injected intraperitoneally with 100 ml of sterile salt solution according to the same schedule. In addition, since the crude mold filtrate contained 1:10000 merthiolate as a preservative and since LoGrippo *et al.*(4) have shown that certain mercury compounds affect the excretion of a mouse encephalomyelitis virus, controls in one experiment were treated with 1:10000 merthiolate in sterile saline. In both *in vivo*(2) and *in vitro*(3) tests M-8450 appeared to exert maximum antiviral effect when given before virus. Therefore in the fourth experiment the total dose of M-8450 was reduced to 75 ml, divided into 3 equal quantities which were administered either before or after virus inoculation. One group of treated monkeys received 2 doses the day before and the third dose 4 hours before virus inoculation, while the other group received the first dose 4 hours after virus inoculation and the remaining 2 doses the following day.

Results. Results of all the tests are given in Table I. These data indicate that M-8450 exerts a distinct antiviral action in monkeys infected with Type 1 poliomyelitis. In the first 2 experiments only 2 out of 14 treated monkeys developed poliomyelitis while 8 of 15 control monkeys became paralyzed. In addition, the incubation period in the treated animals was longer. In the third test half of the treated monkeys received an additional dose of M-8450 14 days after virus inoculation to determine if the paralysis seen in the first experiment could be prevented. This treatment did not appear to have any bene-

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[†] Supplied through the courtesy of Dr. W. S. Boniece and Dr. C. G. Culbertson, of the Lilly Research Laboratories, Indianapolis, Ind.

TABLE I. Effect of M-8450 on Poliomyelitis in Cynomolgus Monkeys Infected Subcutaneously with 10 PD_{50}'s of Poliomyelitis Virus.

Exp.	Treatment	Day of onset of paralysis	
		Treated	Control
1	25 ml, ×3 before, ×1 after virus	24, 30, S, S, S, S, S, S, S, –*	11, 13, 18, 21, 32, S, S, S, S, S†
2	Same as 1	S, S, S, S, S	10, 20, 24, S, S
3	" " "	42, S, S, S, S	7, 10, 13, 13, S‡
	" " " + 25 ml ×2, 14 days after virus	28, S, S, S, S	12, 29, S, S, S†
4	25 ml, ×3 before virus	22, 22, 23, S, S	8, 8, 11, 14, S
	25 " " after "	12, 15, S, S, S	

* Died 6th day due to bacterial infection. † Controls treated with saline. ‡ Controls treated with merthiolate.

ficial effect. That merthiolate *per se* does not contribute to the antiviral action of the M-8450 is shown by the fact that 4 of 5 merthiolate-treated controls developed paralysis as compared to 2 of the 5 saline controls. Administration of a smaller amount of M-8450, *i.e.* 75 ml either before or after virus inoculation, appeared to be less effective as 3 of 5 and 2 of 5 of the monkeys in the treated groups developed poliomyelitis compared to 4 of 5 in the control group. Although the differences in morbidity between the pre-inoculation and post-inoculation treatment groups are not marked, the somewhat longer interval to the onset of paralysis in the group of monkeys receiving M-8450 before virus inoculation suggests that pre-treatment might prove to be the more effective. Combining the results of all experiments in which monkeys received 100 ml or more of M-8450, only 4 of 24 treated monkeys developed poliomyelitis, the day of onset of paralysis ranging from 24 to 42 days, while 14 of 25 virus control monkeys became paralyzed in 7 to 32 days.

Discussion. These studies show that the crude filtrate of a penicillium mold has an antiviral action in monkeys infected subcutaneously with Type 1 poliomyelitis. Previously other investigators had reported its action against Type 2 poliomyelitis in mice(2) and against the 3 types of poliomyelitis in tissue culture(3). Although the relatively large volume of the crude filtrate that was employed contained 1:10000 merthiolate, this did not appear to be responsible for any of the antiviral activity observed with M-8450 and merthiolate treatment alone had no antiviral effect.

Pre-treatment with M-8450 conferred marked protection against poliomyelitis both by reducing the incidence of paralysis in monkeys and by increasing the incubation period in those animals finally developing paralysis. The probability of chance occurrence of this difference in morbidity is less than 5 times in 1000.‡ The prolongation of incubation period is even more marked. Statistical evaluation of this incubation period is complicated by the presence of survivors but may be accomplished by employing the reciprocal of the incubation period and using O as the reciprocal incubation period for a survivor, as suggested by Gaddum(5). Using this procedure the mean reciprocal incubation period of the treated group was 0.0056 day^{-1} and of the control group 0.0403 day^{-1}. Such a difference in mean reciprocal incubation periods would be expected to occur as a chance phenomenon less than one time in 10,000 similar replications, and statistically the incubation period of the treated group was concluded to be significantly greater than that of the control group.

Additional experiments would be required to demonstrate that treatment with 75 ml of M-8450 is much less effective than 100 ml although these experiments suggest that trend. While the numbers of monkeys developing poliomyelitis after treatment with the lesser amount are not significantly different statistically the results tend to be poorer. The relative prophylactic and therapeutic effectiveness of M-8450 in monkeys also requires additional investigation although it appears that its antiviral activity is greater when administered before virus, in agreement with the findings in mice(2) and in tissue culture(3). In its present state of development M-8450 does not appear to have direct therapeutic application. However, its effectiveness in both preventing infection with and prolonging the incubation period of poliomyelitis in monkeys seems to justify further investigation of its properties and mechanism of action.

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‡ The authors are indebted to Dr. F. M. Hemphill, Department of Public Health Statistics, School of Public Health, University of Michigan, for guidance in the statistical evaluation of these data.

Summary. The crude filtrate of a penicillium mold, M-8450, was shown to have antiviral action in cynomolgus monkeys inoculated subcutaneously with the Mahoney strain of Type 1 poliomyelitis. Treatment with at least 100 ml of this crude filtrate reduced the morbidity and increased the incubation period of infected animals. Less intensive treatment appeared to be less effective. The antiviral action of this substance appears to merit further study.

1. Powell, H. M., Culbertson, C. G., McGuire, J. M., Hoehn, M. M., and Baker, J. A., *Antibiotics and Chemotherapy,* 1952, v2, 432.

2. Powell, H. M., and Culbertson, C. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 161.

3. Hull, R. N., and Lavelle, J. M., *ibid.,* 1953, v83, 787.

4. LoGrippo, G. A., Earle, D. P., Jr., Brodie, B. B., Graef, I., Bowman, R., and Ward, R., *Am. J. Hyg.,* 1952, v55, 55.

5. Gaddum, J. H., *Pharmacol. Rev.,* 1953, v5, 87.

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Presence of 17-Ketosteroids in Adrenal Perfusates.* (20800)

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Studies on the nature and effects of adrenocortical steroids have indicated that the adrenal cortex synthesizes C_{19} as well as C_{21} steroids. Reichstein and coworkers isolated Δ^4-androstene-3,17-dione(1), Δ^4-androstene-3,11,17-trione (adrenosterone)(2), and androstane-3β,11β-diol-17-one(3) from adrenal extracts. Cow adrenal venous blood was shown by Gassner *et al.* to contain an androgenic steroid characterized as an α,β unsaturated, hydroxylated, 17-ketosteroid(4). Bush reported the possible presence of Δ^4-androstene-11β-ol-3,17-dione in the adrenal venous blood of rats, sheep and cats(5), but more recently considers his compound to be 17-hydroxyprogesterone(6). In the systematic investigation of steroidal compounds obtained during adrenal perfusions Hechter and his group found several Zimmermann reactive zones on chromatograms run on the perfusates(7). Recently Δ^4-androstene-11β-ol-3,17-dione has been identified in human adrenal venous blood(8) and urine(9) after ACTH administration. The data presented here represents a preliminary report on the presence of Δ^4-androstene-3,17-dione, Δ^4-androstene-3,11,17-trione, and Δ^4-androstene-11β-ol-3,17-dione in blood perfused through beef adrenals.

Experimental. Six liters of citrated beef blood, to which 120,000 units penicillin and 0.6 g streptomycin had been added, were perfused through 6 beef adrenals for 5 cycles as described by Hechter and coworkers(10). After the first cycle, 25 mg ACTH (Armour) was dissolved in propylene glycol-saline and infused into blood over 3 hours. Acetic acid-1-C^{14} was added to the blood prior to perfusion in one experiment (perfusion no. 5). A total of 5 perfusions was conducted. The perfusates were extracted with 3 portions of twice the volume of redistilled ethyl acetate by gently shaking the blood and solvent together(11). The extracts were evaporated to dryness under

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reduced pressure; the residues partitioned between 90% methanol and pentane, and the residues from the methanolic fractions transferred to diethyl ether.

In perfusions 1 and 5, the ether fractions were washed with water, dried over anhydrous Na_2SO_4, and brought to dryness. Separation of the neutral fractions into ketonic and non-ketonic fractions was carried out with the Girard T reagent. The ketonic fractions were further fractionated to characterize individual steroids. The material in the ether fractions from perfusions 2, 3, and 4 was transferred into benzene and fractionated by silica-gel chromatography(12). Appropriate fractions were analyzed qualitatively by paper chromatography and quantitatively by the Zimmermann reaction for the 17-ketosteroids.

Aliquots were taken from all samples for total 17-ketosteroid determinations. The remainder was placed on propylene-glycol impregnated Whatman No. 1 filter paper strips and chromatographed twice for 12-48 hours in the ligroin-propylene glycol solvent system (13). The location of individual compounds was determined by examination of the paper under ultraviolet light, and reacting the paper strips with a) the Zimmermann reagent and b) 2,4-dinitrophenylhydrazine in 1 N HCl solution(14). The Zimmerman reactive zones of the paper chromatograms from perfusate No. 5 were subjected to further analysis. The individual steroids were eluted from paper with redistilled methanol and dried. Employing paper chromatographic mobility as the criterion for identification, the eluates were chromatographed a) as the free compounds on propylene glycol impregnated paper in the solvent system ligroin-benzene (1:1), b) as the hydrazone derivatives, formed after reacting with Girard reagent T, in the system butanol-water (84:16)(14), c) as the acetate derivatives, formed after reacting the eluates with acetic anhydride in pyridine(15), in the solvent system ligroin-ethylene glycol phenyl ether(16), and d) as the free compounds after oxidation with chromic acid(17) in the ligroin-propylene glycol solvent system. The absorption curve between 220 and 500 mμ of the chromogens produced by treatment of the eluates with concentrated H_2SO_4(18) was also obtained.

Three blood control perfusion experiments were done. In control experiment 1, 10 l of blood with added C^{14}-labeled acetate circulated through the perfusion apparatus for 3 hours. The blood was then extracted with ethyl acetate, partitioned between pentane and 90% methanol, and tested for Zimmermann reactive material as described above. In control Experiment 2, 10 mg each of corticosterone (Kendall's Cpd. B) and 11-desoxy-17-hydroxy-corticosterone (Reichstein's Cpd. S), were mixed with 1.3 l of citrated blood. Similarly, 10 mg each of 17-hydroxycorticosterone (Kendall's Cpd. F) and 11-dehydro-17-hydroxycorticosterone (Kendall's Cpd. E) were mixed with 1.5 l of blood in control No. 3. The 2 blood samples were extracted immediately with ethyl acetate, partitioned between 90% methanol and pentane, separated into ketonic and non-ketonic fractions, and the ketonic residue chromatographed on paper. Individual Zimmermann reactive zones were eluted from paper and their Zimmermann reactive material determined quantitatively.

Results. The perfusates contained between 0.35 and 1.5 mg total 17-ketosteroids. The data suggest that silica-gel chromatography results in greater purification of steroid extracts than the procedure involving Girard separation. Recovery experiments, in which 1 mg of Δ^4-androstene-3,17-dione was added to 2 l of blood, in duplicate, resulted in 63% and 68% recovery. The ratio of Zimmermann reactive substances to formaldehydrogenic substances in blood varied between 1:35 and 1:50.

Paper chromatography in ligroin-propylene glycol of the five perfusates gave 9 distinct Zimmermann reactive zones. In Table I, the relative mobilities (R_T) (mobility relative to androsterone = 1.00 cm/hr), the colors produced upon reaction with the Zimmermann reagent and 2,4-dinitrophenylhydrazine, and the presence or absence of ultraviolet light absorption by the reactive areas are listed. Only those zones with identical R_T values found in two or more perfusates are included in Table I. Except in the case of the rapidly moving compound I, the R_T values among

TABLE I. Relative Mobilities (R_T) of Compounds I-V and Standards in Ligroin-Propylene Glycol.

Compounds in perfusates	No. of perfusates containing compounds	R_T	Ultra-violet absorption	Color reaction Zimm.	Color reaction DNPH
I	2	2.3, 2.8*	—	B-P	Y
II	3	1.70	+	P	Or
III	4	.42	+	P	Or
IV	4	.10	+	P	Or
V	2	.083	—	B	—
Standards					
androstane-3,17-dione		2.5*	—	P	Y
Δ^4-androstene-3,17-dione		1.70	+	P	Or
Δ^4-androstene-3,11,17-trione		.45	+	P	Or
Δ^4-androstene-11β-ol-3,17-dione		.10	+	P	Or

* Mobility of androstane-3,17-dione in ligroin-propylene glycol varies by 25% from the mean.
The following abbreviations are used: Zimm. = Zimmermann reagent, DNPH = 2,4-dinitrophenylhydrazine in 1 N HCl, B = blue, P = purple, Y = yellow, Or = orange.

TABLE II. Mobilities of Compounds I-IV from Perfusion 5 and Their Derivatives in Various Solvent Systems.

Compound	Free compound	Derivative: Girard hydrazone	Derivative: CrO_3 oxidation product
	Solvent system: Ligroin-Bz (1:1)-Prop. G.	Solvent system: Butanol-water (84:16)	Solvent system: Ligroin-Prop. G.
	Mobility (cm/hr)		
I	—	.83	—
II	—	.14	—
III	.43	.08	.47
IV	.17	.06	.42
Δ^4-androstene-11β-ol-3,17-dione	.15	—	.10
Δ^4-androstene-3,11,17-trione	.53	.07	.41
Δ^4-androstene-3,17-dione	—	.15	1.65
testosterone	.58	.85	—
Δ^4-androstene-3-one	—	.98	—

The following abbreviations are used: Bz = benzene, Prop. G. = propylene glycol.

replicate samples differed by 10% or less. These paper tests indicated that all compounds contained a ketone group at carbon atoms 3 and 17 and α,β unsaturation in the structure of compounds II, III and IV. Interfering material eluted from paper with the individual compounds prevented their spectrophotometric determination at 240 mμ.

Five Zimmermann reactive compounds were found in perfusate No. 5 (perfusion with C^{14}-labeled acetate). Compounds I to IV, when subjected to different conditions and treatment, exhibited characteristic mobilities which are presented in Table II. The mobilities of the trimethylammoniumacethydrazone derivatives of compounds I and II were identical with those of testosterone and Δ^4-androstene-3,17-dione, respectively. The reaction product of compounds I and II with acetic anhydride in pyridine did not move appreciably on filter paper in the system ligroin-ethylene glycol phenyl ether during a 21-hour period. Testosterone acetate and androsterone acetate moved 0.24 and 0.50 cm/hr, respectively, in the same system, indicating that no monoacetates had been formed. The rates of movement of compounds III and IV in the ligroin-benzene (1:1)-propylene glycol system corresponded to the mobilities of the standards

Δ^4-androstene-3,11,17-trione and Δ^4-androstene-11β-ol-3,17-dione, respectively. The hydrazones of both compounds III and IV exhibited a mobility identical with Δ^4-androstene-11-one-3,17-dihydrazone. Oxidation of compounds III and IV with chromic acid and chromatography of the oxidation products gave a single Zimmermann reactive area with an R_T value of 0.45 ± 0.03 (ligroin-propylene glycol system) corresponding to the mobility of Δ^4-androstene-3,11,17-trione. In all instances, the Zimmermann reactive and K_2PtI_6 reactive zones on paper were found to be radioactive. Determinations of specific activities were not possible because the variable weight of the non-steroidal portion of the methanol eluate frequently exceeded that of the steroidal portion. Nevertheless, when consecutive tests were carried out on one aliquot of an eluate, the activity did not change significantly.

The absorption curve of the chromogen of compound I in concentrated H_2SO_4 could not be identified; that of compound II exhibited a minimum at 240 mμ and a maximum at 310 mμ. The spectra of both the crystalline Δ^4-androstene-3,17-dione and androstane-3,17-dione chromogens contained one minimum at 240 mμ and one maximum at 300 mμ.

An aliquot of labeled compound III (contained in 6.6 mg of eluted material) was diluted with 2.0 mg of crystalline, non-labeled Δ^4-androstene-3,11,17-trione. This sample was chromatographed 4 times in the ligroin-propylene glycol system, once in the ligroin-benzene (1:1)-propylene glycol system, and once as the dihydrazone derivative in butanol-water. The C^{14} activity remained associated with the Zimmermann or K_2PtI_6 reactive zone in every chromatogram. The infra-red absorption spectrum of the diluted compound III was identical with Δ^4-androstene-3,11,17-trione.

The purified perfusate of control experiment 1 contained less than 50 μg of total 17-ketosteroids. No Zimmermann reactive zones were noted on the paper chromatograms. The chromatograms of the residues from control Experiments 2 (Compounds B and S added to blood) and 3 (added Compounds E and F) contained one Zimmermann reactive zone each with respective R_T values of 1.00 and 0.41. These two 17-ketosteroids were found quantitatively as contaminants of the crystalline Compounds S and E added to blood in the control experiments.

Discussion. Characterization of Zimmermann reactive substances found in the adrenal perfusates required use of highly sensitive analytical methods because of the small quantities of material present (30-250 μg per Zimmermann reactive zone). Paper chromatography meets this requirement. Identification of compounds I to IV was based primarily on the chromatographic behavior of the free compounds and their derivatives in different solvent systems, and by the use of several color tests. Although identification by these methods is not as definitive as by classical procedures (melting point of free steroids and derivatives, infrared absorption curve, optical rotation), the correctness of the identifications made on the basis of the sum of the results obtained from the 4-5 tests used in this investigation carries a significant degree of certainty.

The color reactions and mobility of the free compound I indicated a saturated 3,17-diketone, possibly androstane-3,17-dione or etiocholane-3,17-dione. Formation of a monohydrazone and the presence of a non-reactive polar group, which also did not form an acetate, added to the evidence for the probable nature of compound I. Similarly, employing the same criteria and tests, compound II and Δ^4-androstene-3,17-dione gave identical reactions. The excellent reproducibility of the mobility of Δ^4-androstene-3,17-dione in ligroin-propylene glycol as compared to androstane-3,17-dione and the added parameter of H_2SO_4 chromogen absorption curves, which agreed within experimental error, allow compound II to be identified as Δ^4-androstene-3,17-dione with a high degree of certainty.

The behavior of compounds III and IV indicated both to be Δ^4-3,17-diketones containing a third oxygen function which did not form an acetate or hydrazone. Compound III was not affected by chromic acid oxidation while compound IV was converted to compound III. These results indicate that compound III has a carbonyl group and compound

IV a β-hydroxyl group attached to C-11, neither of which would react with acetic acid in pyridine or Girard reagent T. The mobilities of the free compounds III and IV in the two solvent systems employed were identical with Δ^4-androstene-3,11,17-trione and Δ^4-androstene-11β-ol-3,17-dione, respectively. The results with the mixture of non-labeled Δ^4-androstene-3,11,17-trione and labeled compound III further strengthen the conclusion that these two substances are identical.

Compound V was present in insufficient amounts to attempt its characterization. Its mobility and ultraviolet light absorption would suggest a C_{19} steroid with three oxygen functions including a Δ^4-3-ketone.

The large amounts of formaldehydrogenic material found in the perfusates compared to the amounts of 17-ketosteroids suggested the possibility that compounds II, III and IV were chemical degradation products of the C_{21} steroids formed by the adrenal gland during perfusions. The absence of Δ^4-androstene-11β-ol-3,17-dione, Δ^4-androstene-3,17-dione and Δ^4-androstene-3,11,17-trione in the control samples containing 10 mg each of Compounds B, S, E and F which agrees with the absence of Δ^4-androstene-3,11,17-trione in blood from cortisone perfused adrenal glands (11) strongly suggests that these three 17-ketosteroids were not formed during the course of purifying and analyzing the perfusates. Rather, they are of adrenal origin.

Summary. Bovine adrenal glands were perfused with blood containing added ACTH and the perfusates purified for study of their C_{19} steroid contents. Individual steroids present in the purified extracts were detected and characterized by studying the behavior of the free compounds and their derivatives by paper chromatographic techniques involving several solvent systems and color tests. By these methods, five 17-ketosteroids were found in adrenal perfusates, three of which exhibited properties identical to standard samples of Δ^4-androstene-3,17-dione, Δ^4-androstene-3,11,17-trione and Δ^4-androstene-11β-ol-3,17-dione. The conclusion is reached that the methods used here allow the identification of these three steroids in adrenal perfusates with a high degree of certainty.

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1. Reichstein, T., and v. Euwe, J., *Helv. Chim. Acta*, 1941, v24, 879.

2. Reichstein, T., *ibid.*, 1936, v19, 29, 223.

3. Reichstein, T., and v. Euwe, J., *ibid.*, 1938, v21, 1197.

4. Gassner, F. X., Nelson, D. H., Reich, H., Rapola, R. T., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 829.

5. Bush, I. E., *J. Endocrinol.*, 1953, v9, 95.

6. ———, Josiah Macy Jr., Foundation Conf., 1952, vIV, 118.

7. Hechter, O., unpublished work.

8. Romanoff, E. B., Pincus, G., and Hudson, P., *J. Clin. Endocrinol. and Metab.*, 1953, v13, 1546.

9. Salamon, I. I., and Dobriner, K., *J. Biol. Chem.*, 1953, v204, 487.

10. Hechter, O., Jacobsen, R. P., Schenker, V., Levy, H., Jeanloz, R. W., Marshall, C. W., and Pincus, G., *Endocrinol.*, 1953, v52, 679.

11. Meyer, A. S., *J. Biol. Chem.*, 1953, v203, 463.

12. Levy, H., Jeanloz, R. W., Marshall, C. W., Jacobsen, R. P., Hechter, O., Schenker, V., and Pincus, G., *J. Biol. Chem.*, 1953, v203, 433.

13. Savard, K., *ibid.*, 1953, v202, 457.

14. Zaffaroni, A., Burton, R. B., and Keutman, E. H., *Science*, 1950, v111, 6.

15. ———, *J. Biol. Chem.*, 1949, v177, 109.

16. Burton, R. B., Zaffaroni, A., and Keutman, E. H., *J. Biol. Chem.*, 1951, v193, 769.

17. Neher, R., and Wettstein, A., *Helv. Chim. Acta*, 1952, v35, 276.

18. Zaffaroni, A., *J. Am. Chem. Soc.*, 1950, v72, 3828.

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A Recording Digital Calorimeter.* (20801)

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The digital calorimeter, as originally described(1,2), consisted of an aluminum calorimeter cup, set in broken cork, and covered by a rubber membrane with apertures for the finger, a stirring device and a Beckmann thermometer. To measure the calories elaborated by the digit per unit of time, the calorimeter was usually set at 6 or 7°C below the temperature of the arterial blood. The rise in temperature produced by the digit, however, was modified by loss of heat from calorimeter to room because of the difference between their respective temperatures. This loss, therefore, was measured with a cork replacing the digit and was added to the temperature rise produced in the calorimeter by the digit. The total was considered representative of the total heat output of the finger or toe.

Although this procedure is sound for the measurement of calories within the error of the method as a whole, it involves 2 sets of measurements under slightly different conditions. Room temperature and the rate of stirring, for example, must be the same in the 2 phases of the procedure. Another objection has also been raised to the effect that a variable cooling lag might introduce error(3) although this is probably of more significance for the hand calorimeter than for the smaller digital calorimeter. To meet this objection, Greenfield *et al.*(4) introduced a calorimeter incorporating a stirred electrically heated water jacket, the temperature of which could be regulated to keep the temperature of the water within the calorimeter constant despite varying room temperature. Thus, the contribution made by the digit could be measured directly. This instrument, however, contained a liter of water and was therefore too large and unwieldy for practical digital calorimetry using one finger tip. Wilson(5) balanced heat lost from his calorimeter to the room by heating the outside of the cup with an electric bulb regulated by a rheostat. This simple device works well but has the disadvantage of bright lighting and a tendency to heat the hand or foot as well as the calorimeter cup.

Heat loss to the ambient air can also be balanced by a heating wire inserted into the cup.† This heating wire is connected to the secondary winding of a step-down transformer with an ammeter in series. The voltage of the primary winding of the transformer is controlled by a variable autotransformer, fed by a 120 volt, 60 cycles/sec. line. The heating wire is 2½ feet long and has a total resistance of 11.5 ohms. It is coiled and ensheathed in polyethylene tubing of appropriate size. The maximum voltage applied to the wire is 10 volts. This corresponds to a maximum heat input into the calorimeter by the wire of 120 g cal./min. By adjusting the voltage in the primary winding of the step-down transformer, the current in the wire and hence the heat input can be changed and the temperature of the stirred water in the calorimeter can be kept constant within 0.01°C. The actual heat input required to keep temperature constant varies from 0 to 5 g cal./min. depending on ambient temperature. The position of the wire must be adjusted to keep it free of the digit, the stirring bar and the thermometer itself.

Several other changes are incorporated in this calorimeter (Fig. 1) which increase ease of operation. The cup for the finger contains 125 cc, and for the toe, 250 cc of water. It consists of an evacuated silvered flask, the evacuation bulb being eccentric so that the bottom can be fairly flat. No other insulation is needed and the flask is held in place by a clamp on its metal jacket. Either a rotary or an electromagnetic stirrer may be used. Transfer of heat from the electromag-

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* Aided by a grant from the National Heart Institute, Department of Health, Welfare and Education, Washington, D. C.

† This principle was used in a hand calorimeter by Greenfield and Scarborough(3).

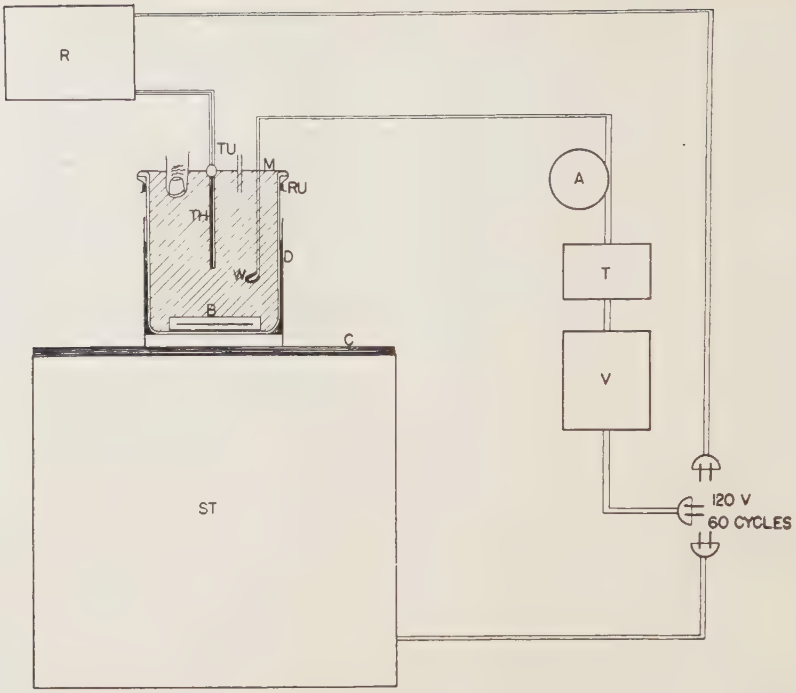

FIG. 1. Diagram of recording digital calorimeter. V, variable voltage control; T, step down transformer; A, milliammeter; R, recorder (Leeds-Northrup #61,452); Tu, glass tube; M, rubber membrane; Ru, rubber band; Th, platinum resistance thermometer (Beckmann thermometer can be substituted if no record is required); W, resistance wire ensheathed in polyethylene tubing; B, plastic stirring bar; D, silvered, evacuated, double-walled, glass calorimeter cup containing water. Cemented to the cup is a metal sheath, only the edge of which stands on the cork slab beneath. A clamp and ring stand (not shown in the diagram) hold the unit in place on the stirrer. C, cork slab; St, electro-magnetic stirrer (large).

net is prevented by a cork slab between the housing of the magnet and the edge of the metal jacket of the cup. This heat, as well as the heat of stirring produces no error, since adjustment is always made to near zero via the heating wire. In addition, a record is made with a cork before and after the digital record. Any deviation from zero is averaged and either added or subtracted from the digital slope. An air-vent which can be water-sealed is incorporated in order to make the insertion of the finger and the addition of water easier. A Beckmann thermometer or a non-adjustable mercury thermometer, registering with an accuracy of ±0.01 °C over the desired temperature range may be used with this apparatus. A platinum resistance thermometer and Leeds-Northrup recorder provide a permanent record of the temperature changes. A typical record, the slope of which is accurate to ±0.01 °C is demonstrated

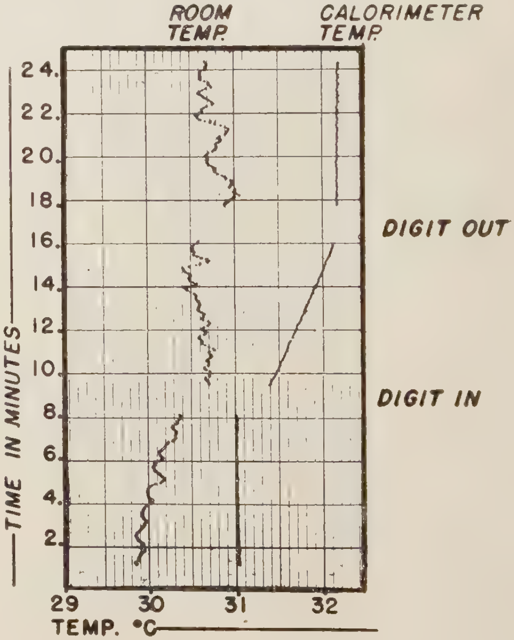

FIG. 2. A typical record of digital calorimetry.

in Fig. 2. With a constant heat input via the resistance wire, the rise in temperature as measured by the Beckmann thermometer is exactly the same as that measured by the corrected slope of the recorded temperature.

The hydrothermic equivalent of the apparatus was calculated as follows: With a constant heat input at a given calorimeter temperature under fixed room temperature conditions, the calories engendered in distilled water were equated with those engendered in potassium carbonate solution of known specific heat. The only unknown, the hydrothermic equivalent of the apparatus can be calculated(6). It was 5 ± 1 cc for the digital, and 14 ± 2 cc for the hallucal calorimeter.

Summary. A recording digital calorimeter is described.

The advice of Dr. S. Feitelberg and the technical assistance of Miss P. R. Cohen, Mr. H. Rothman and Mr. S. Roth are gratefully acknowledged.

1. Mendlowitz, M., *Clin. Sci.*, 1938, v3, 387.

2. ———, *Angiology,* 1950, v1, 247.

3. Greenfield, A. D. M., and Scarborough, H., *Clin. Sci.,* 1949, v8, 211.

4. Greenfield, A. D. M., and Shepherd, J. T., *Clin. Sci.,* 1950, v9, 323.

5. Wilson, G. M., *Quart. J. Med., N. S.,* 1952, v21, 201.

6. Mendlowitz, M., *Science,* 1948, v107, 97.

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An Improved Technic for Liquid Emulsion Autoradiography.* (20802)

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The increasing application of radioactive elements as tracers in biological and medical problems and the necessity for more precise information regarding the effects of radioactive elements on tissues have resulted in continuous improvement in autoradiographic procedures for the detailed study of the distribution of these elements. It is generally recognized that autoradiographic resolution on a cytologic scale can be improved by the elimination of all space separating the emulsion from the tissue, the use of thin layers of emulsion, and the use of thin tissue sections (1-4). In the course of a study of the deposition of long-lived radioactive elements at the cellular level, it has been necessary to improve and modify existing technics for the preparation of liquid nuclear track emulsions for autoradiography. As a result, an autoradiographic procedure has been developed which eliminates all emulsion-tissue separation and allows the preparation of emulsions less than one-half micron in thickness.

Materials and methods. All tissues are fixed in acetone at room temperature to minimize migration of bone seeking radioisotopes and fogging of emulsions by chemical constituents. Tissue sections, 2 to 5 microns thick, are cut in an embedding medium such as celloidin, paraffin, or plasticized nitrocellulose(5), and mounted on glass slides with egg albumin or other fixatives. (For very hard tissues that tend to curl, *e.g.,* undecalcified bone, it is advisable to roll the damp section on a slide that has been previously coated with 1.5% gelatin, drained, and air-dried.) The embedding medium is then dissolved in a suitable solvent, *e.g.,* xylene for paraffin, ether alcohol for celloidin, and ether alcohol or acetone for the plasticized nitrocellulose, and the tissue section is dried. NTA and NTB liquid nuclear track emulsions in the solid state in air- and light-tight, Dewar flasks, and maintained at 5°C, were kindly supplied for test purposes by the Eastman Kodak Co. NTB emulsion has a high sensitivity, producing short tracks for low energy beta particles or electrons. NTA emulsion has a low sensitivity, responding quite slowly to beta irradiation. Both emulsions are capable of forming alpha tracks.

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* This work was begun at Argonne National Laboratory while Dr. Arnold held an Atomic Energy Commission post-doctoral fellowship administered by the National Research Council; later phases were carried out under partial support by the Atomic Energy Commission.

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A small quantity of the gelatinous emulsion is transferred on a clean glass or stainless steel spatula from the Dewar storage flask to a beaker that has been placed in a constant-temperature water bath at 38°C. Melting occurs in 15 to 25 minutes, depending on the amount of emulsion to be used. When the emulsion has reached the liquid state, it is applied to the mounted tissues by means of a soft, clean No. 6 or No. 7 camel's hair brush. The thickness of the emulsion layer is easily controlled by varying the amount of emulsion on the brush. The sweep of the brush should neither start nor stop over the tissue section. With experience, as many as 50 slides may be satisfactorily covered in 5 minutes.

The emulsion coating will gelatinize at room temperature in about 5 minutes. If the emulsion tends to clump on the tissue, the slides should be cooled on a metal plate above an ice bath before coating. After the emulsion coating has gelatinized, the slides are dried in a gentle current of dust-filtered cool air (that may be obtained from an electric fan with a cheese cloth filter). The slides are then stored for exposure in a plastic slide box containing an open vessel of saturated calcium chloride solution. The slide box is sealed so that it it both light- and air-tight and placed in a refrigerator at 0° to 5°C for the desired exposure period which, as in any autoradiographic procedure, depends on the amount of radioactivity present, the distribution of the radioactive isotope in the tissue, the degree of image intensity desired, and the half-life of the radioisotope(1,2,4). All solutions used in the developing process are kept at 18°C to prevent softening and liquefaction of the thin emulsion. The slides are processed in a horizontal position to further minimize distortion. To prevent the accumulation of bubbles beneath the thin emulsion, all solutions are degassed by reducing pressure to the boiling point at room temperature (using a simple water aspirator and vacuum flasks) and all washing steps are eliminated. The slides are held for one minute in D-19 developer (Eastman Kodak) and transferred to acid fixer with hardener for 2 minutes. The slides are then placed in 4% formaldehyde solution with occasional gentle agitation for 5 minutes and then transferred to a second 4% formaldehyde solution for 4 hours.

Staining. After the emulsion has been hardened in formalin, the tissues may be stained at room temperature with hematoxylin and eosin without fear of softening the emulsion. The best results are obtained by overstaining the slides in Mayer's hematoxylin and then differentiating in 5% ammonium alum solution until the gelatin of the film is clear. (When preparing undecalcified bone autoradiographs, the sections should be decalcified prior to staining in 5% ammonium alum, which does not attack the silver grains.) The slides are then placed in ammonia water at a pH of 8.5 for 30 minutes. (A higher pH tends to soften and distort emulsions and a lower pH fails to protect against residual thiosulfate attacking the silver image.) The tissues are rinsed for a short time in distilled water and are then stained in aqueous eosin (½% for 30 to 60 seconds). The gelatin is cleared of eosin by vigorous differentiation in 50-90% alcohol for 10 to 15 minutes, and the sections are mounted in clarite or permamount in the usual manner. If a few bubbles of gas should persist beneath a section, they may be removed by soaking for 12 hours in a 1:1 dilution of the mounting medium(6). The autoradiographs are examined under ordinary transmitted light or with a dark field microscope.

Discussion. The production of thin emulsion layers and the elimination of tissue emulsion interspace are theoretical as well as practical advantages in the improvements of autoradiographic resolution(1-4). Fig. 1 is a microphotograph of an autoradiograph and 4 microns thick section of a trabecular bone from a rat sacrificed one week following Ca^{45} administration. The line of increased grain density running down the center of the structure overlays Ca^{45} incorporated in the under-

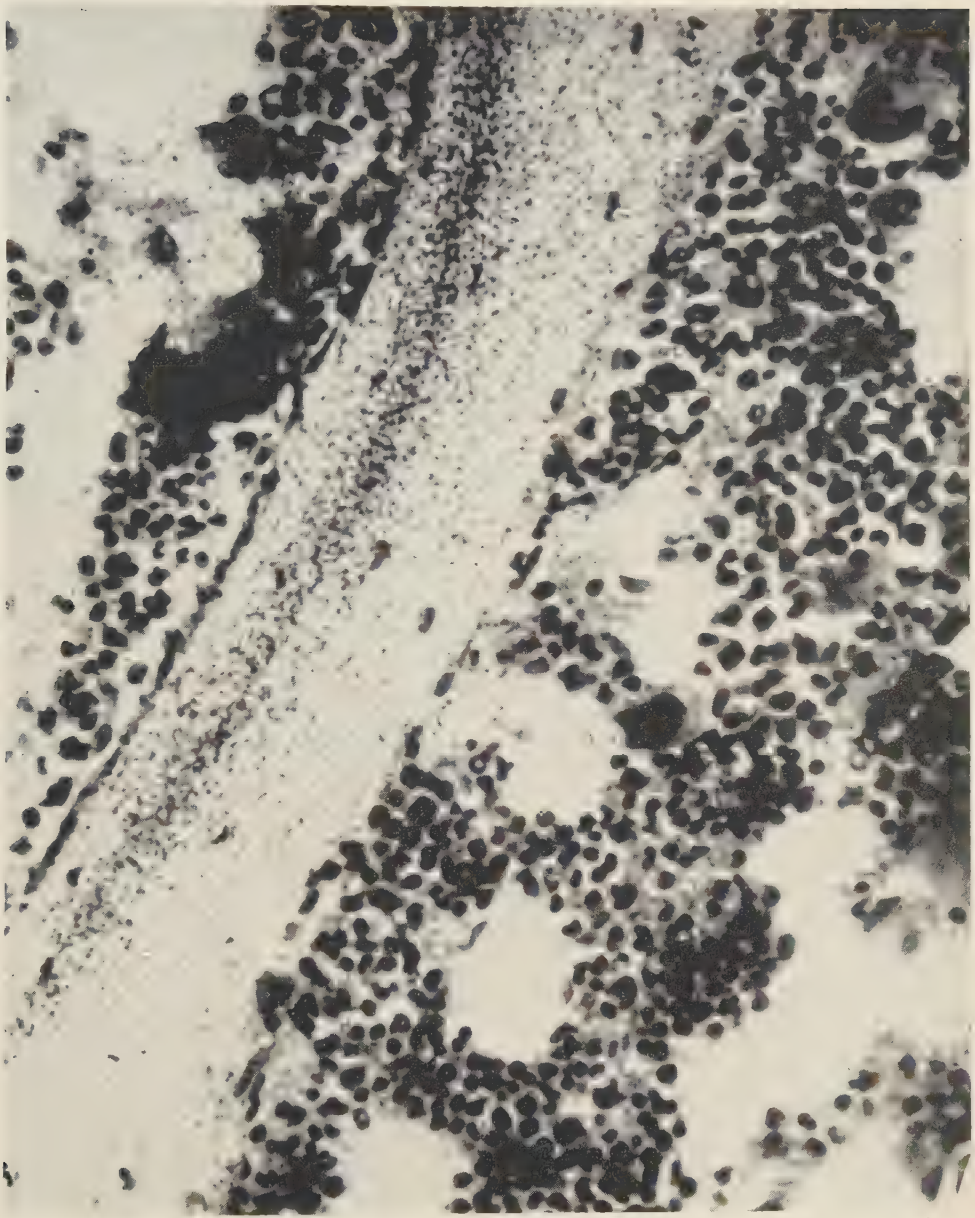

FIG. 1. Microphotograph of an autoradiograph and 4 μ thick section of a trabecular bone from a rat sacrificed 1 wk following Ca^{45} administration.

lying bone, which was deposited one week prior to sacrifice. The osteocytes and various cells of the marrow may be compared to the width of the line of increased grain density. It is apparent that this line is no wider than 14 microns in the center. However, no accurate estimation of the absolute resolution of the autogram is possible because the width of the strip of bone containing the increased Ca^{45} concentration is not determinable.

In addition to considerations of resolution, the thin emulsion layer has a distinct advantage over thick ones in high magnification microscopic study and photomicroscopic reproduction of detailed autoradiographs. The depth of focus of most microscope objectives with a 20× or greater magnification is less than 3 microns. When a histological section and intimately superimposed autoradiographs are viewed simultaneously one or the other tends to be out of focus. Because of this limitation of the objective depth of focus, any diminution in emulsion thickness, section thickness or emulsion tissue interspace will facilitate study of the high resolution autoradiogram.

Several problems arose during the course of the development of the described technic which are peculiar to extremely thin emulsion layers of liquid-coated autoradiographs. The first of these is the adherence of the emulsion to tissue in an even layer. Addition of wetting agents, precooling of slides, and dilution of emulsion(2,6) all tend to minimize the tendency of the emulsion to clump; however, occasionally all of these procedures fail on certain preparations for undetermined reasons.

Thin layers of emulsion are subject to cracking when stored at the low humidity produced by most chemical desiccants. When actual cracking of emulsion does not occur, sufficient tension may develop to produce characteristic fine, irregular lines of closely packed silver grains. Emulsion cracking and tension artifacts are virtually eliminated by maintaining a constant relative humidity of 40% during the exposure period. This is simply done by placing an open vessel of saturated calcium chloride solution into the exposure slide box and exposing autograms at 0-5°C.‡

The tendency of bubbles to form between emulsion and tissue during development and fixation procedures was initially a problem. This phenomenon probably occurs in thick as well as thin emulsion preparations; however, the distortion produced by it is much greater in the case of thin emulsion layers. Bubbles began to appear in the developing solution and continued to accumulate throughout all processing steps. The tendency of bubble formation was finally controlled by 1) eliminating all washing procedures, 2) decreasing development and fixation period to a minimum, 3) degassing of all processing solutions prior to use, and finally 4) hardening emulsion in 4% formaldehyde solution. If thin emulsion layers are not hardened in formaldehyde they soften and disintegrate in most aqueous solutions above 20°C.

Chemical fogging of the emulsion has rarely been encountered when processing acetone-fixed tissues. The reason for this is as obscure as the actual mechanism of chemical fogging.

Summary. A method for the preparation of autoradiographs with thin liquid coated emulsion layers has been described.

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‡ Suggested by William P. Norris of Argonne National Laboratory.

1. Doniach, I., and Pelc, S. R., *Brit. J. Radiol.,* 1950, v23, 184.

2. Gross, J., Bogoroch, R., Nadier, N. J., and Leblond, C. P., *Am. J. Roentgenol. Radium Therapy,* 1951, v65, 420.

3. Stevens, G. W. W., *Brit. J. Radiol.,* 1950, v23, 723.

4. Fitzgerald, P. J., Simmel, E., Weinstein, J., and Martin, C., *Laboratory Investigation,* 1953, v2, 181.

5. Arnold, J. S., *Science,* 1951, v114, 178.

6. Bogoroch, R., M. Sc. Dissertation, McGill University, 1950.

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Use of the Trypanosomid Flagellate, *Crithidia fasciculata*, for Evaluating Antimalarials.[*][†] (20803)

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Since the Trypanosomid flagellate, *Crithidia fasciculata* (*Anopheles* strain), a parasite of mosquitoes, requires an exogenous source of folic acid (or closely related compounds) for growth(2) to which it responds quantitatively, it is useful for studying antimetabolites directed at the folic acid group. This strain is not known to have a vertebrate host, but as a member of the Trypanosomidae, it is related to several important intra- and extracellular pathogens.

Earlier investigations in our laboratory with folic acid analogs such as Aminopterin[§] and 10-formyl-folic acid[§] were inconclusive as these compounds had folic acid rather than antimetabolite activity. Several substituted pyrimidines, (structurally more dissimilar to folic acid) among them potent antimalarials (3,4) which also display anti-leukemic activity(5), were next studied. Excellent correlation had been found between the *in vivo* inhibition of *Plasmodium bergei* (in mice) and of *P. gallinaceum* (in chicks)(6) and, in our laboratory, of *in vitro* inhibition of crithidia, by the pyrimidine antimalarials. This contrasts with dissimilar inhibition patterns observed with bacteria and suggests that crithidia, as described here, may be a better guide for preliminary evaluation of antimalarials.

Method. The completely defined medium devised earlier(2) for this strain was modified for these studies (Table I). All cultures were allowed to grow past the logarithmic growth phase, which required between 10-18 days. Growth was measured as optical density on a Welch Densichron. The antimetabolites[||] used

TABLE I. Basal Medium for *Crithidia fasciculata*. Grams (unless otherwise specified) in 100 ml of final medium.

K_3PO_4	.02
Ethylenediamine-tetra-acetic acid (EDTA)	.06
$MgSO_4 \cdot 7H_2O$	.04
Fe (as SO_4)	.1 mg
Ca (as Cl)	.2 mg
Mo (as Na)	.4 mg
Metals mix*	2.5 ml
L-arginine HCl	.025
L-histidine HCl	.02
DL-isoleucine	.005
DL-lysine	.02
DL-leucine	.005
DL-methionine	.008
DL-phenylalanine	.004
DL-tryptophan	.002
L-tyrosine	.005
DL-valine	.005
Triethanolamine	.5
Hemin	2.5 mg
Vit. mix†	1.0 ml
Adenosine‡	3.0 mg
Glucose (aseptically)§	1.0

Folic acid or folinic acid omitted from the base.|| pH 8.1; 2 ml distributed in 10 ml flasks.¶

* 1 ml contains: Zn (as $ZnSO_4$) 1 mg, Mn (as $MnSO_4$) 1 mg, Co (as $CoSO_4 \cdot 7H_2O$) 0.02 mg, Cu (as $CuSO_4 \cdot 5H_2O$) 0.01 mg, B (as H_3BO_3) 0.004 mg, I (as NaI) 0.001 mg, EDTA 1 mg. Concentrations refer to metal ions alone.

† 1 ml contains: Thiamine • HCl 0.6 mg, riboflavin 0.1 mg, Ca pantothenate 0.3 mg, nicotinic acid 0.3 mg, biotin 1.0 μg, pyridoxamine • 2HCl 0.1 mg.

‡ The commercial adenosine used had a 10% impurity of guanosine as determined chromatographically and spectrophotometrically. This impurity did not interfere with this particular study as *Crithidia* can utilize adenosine or guanosine interchangeably(2).

§ One drop of sterile 50% glucose/flask. Preparation: a 50% (w/v) solution of Mallinckrodt C. P. glucose (devoid of traces of yellow impurities found in other C.P. samples from other manufacturers) was brought to pH 3.0-3.6 with H_2SO_4, distributed in 125 × 20 mm screw-capped tubes and autoclaved for 20 min. at 118-120°C.

|| Supplied as "Leucovorin" (Lederle), the Ca • $5H_2O$ salt.

¶ Special "micro-Fernbach" flasks, Kimble catalog No. 26020-S-43.

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[*] Abstract appeared(1).

[†] Aided by grants from Wellcome Research Laboratories, Tuckahoe, N. Y., Lederle Laboratories Division of the American Cyanamid Co., and the Rockefeller Foundation.

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[§] Generously supplied by Dr. H. P. Broquist, Lederle Laboratories, Pearl River, N. Y.

[||] Generously supplied by Dr. G. H. Hitchings, Wellcome Research Laboratories, Tuckahoe, N. Y.

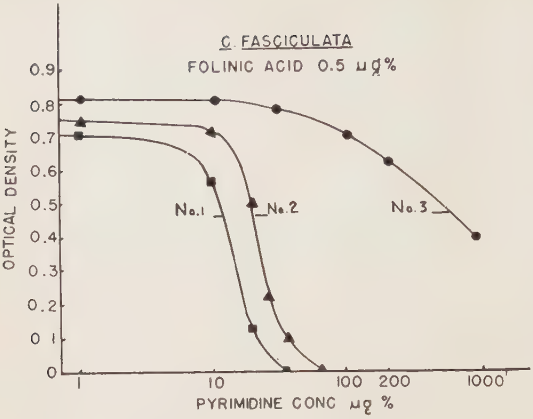

FIG. 1. Graph showing the competitive nature of folinic acid antagonism by 2,4-diaminopyrimidines.

No. 1 5-*p*-chlorophenyl-6-ethyl-2,4 diaminopyrimidine
2 5-(3′4′-dichlorophenyl)-6-methyl-2,4 diaminopyrimidine and
3 5-*p*-chlorophenoxy-6-methyl-2,4 diaminopyrimidine.

were: (1) 5-*p*-chlorophenyl-6-ethyl-, (2) 5-(3′4′-dichlorophenyl)-6-methyl-, and (3) 5-*p*-chlorophenoxy-6-methyl-2,4-diaminopyrimidine. Growth of the organism in the presence of various levels of drug was determined as a function of folic or folinic acid.

Results. Folic and folinic acids are competitively inhibited by these substituted pyrimidines (Table II, Fig. 1). This inhibition of *C. fasciculata* shows patterns closer to the *in vivo* results with plasmodia obtained by the other workers mentioned, and diverges from the results with bacterial test systems. The bacterial systems had failed to distinguish between pyrimidine No. 1 ("Daraprim"), an extremely potent antimalarial, and pyrimidine No. 2 which has only moderate clinical value (Table III).

The failure of quinine or atabrine to antagonize either folic or folinic acid under our experimental conditions indicates, as had been reported(8,9), that these drugs interfere with a system other than the folic acid.

Discussion. The site of action of antifolics is more accessible to analysis in organisms requiring folic acid exogenously. In our unpublished experiments, growth of crithidia in the presence of limiting folic acid was sensitively influenced by other metabolites whose metabolism was linked to that of folic acid, *e.g.*, purine ribosides, thymine, and amino acids such as threonine, methionine, and serine. Details of these interactions will be reported elsewhere.

Since plasmodia requires PABA(10), and all members of the Trypanosomidae thus far studied require folic acid or closely related compounds(11), these requirements may point to vulnerable sites for attack by chemotherapeutic agents. Pathogenic trypanosomes differ widely in sensitivity to drugs. The biochemical differences underlying these species-to-species and strain-to-strain variations in susceptibility to chemotherapeutic agents may have parallels in various non-pathogenic members of this group (such as the strain used in this work) which differ obviously from each other in morphology and nutrition.

The effectiveness of antimalarials is claimed to correlate with their ability to inhibit adenosine-induced auriculoventricular block in rabbits(12). Our preliminary experiments indi-

TABLE II. Antimalarials as Antagonists to Folic Acid in *Crithidia*. Antagonism measured as reduction in final growth (growth expressed as optical density).

		Folic acid	
		0.05 μg %	0.5 μg %
Drug in μg %		Optical density	
No addition		.90	1.
Quinine (calculated as free base)	.1	.94	1.
	1.	1.18	1.
	1000.	1.18	1.
Atabrine (Quinacrine HCl)	.1	.92	1.22
	1.	1.30	1.30
	10.	1.20	1.28
	100.	1.22	1.26
	1000.	1.14	1.32
Pyrimidine No. 1*	1.	.60	.92
	3.	.52	1.
	10.	0	.96
	30.	0	.72
	100.	0	0
" No. 2	1.	.92	.82
	3.	.65	.78
	10.	.33	.76
	30.	0	.74
	100.	0	.74
	300.	0	0
" No. 3	3.	.66	.89
	10.	.62	1.02
	30.	.26	.72
	100.	0	.88
	300.	0	.24
	1000.	0	0

* Commercially known as "Daraprim."

TABLE III. Inhibitions of Various Organisms by Substituted Pyrimidines. Results for each organism are relative with pyrimidine No. 3 arbitrarily set at 1; the other values represent multiples of this standard. See references in Table for experimental detail.

Pyrimidine No.	*Plasmodium bergei*(6)	*Plasmodium gallinaceum*(6)	*Crithidia fasciculata*	*Streptococcus faecalis*(7)	*Lactobacillus casei*(7)	*Leuconostoc citrovorum**(6)
1	285.7	150.	33.3	12.5	0.89	2.7
2	185.7	35.	10.	12.5	1.2	2.7
3	1.0	1.0	1.0	1.0	1.0	1.0

* Felton and Niven(13) showed that "*L. citrovorum*" is actually a typical strain of *Pediococcus cerevisiae*.

cate that effective drugs interfere with the utilization of adenosine by crithidia, for which, under certain conditions, adenosine or guanosine are the only compounds which satisfy the nucleic acid component requirement(2). Whether this unusual specificity of ribosides is linked with the special role assigned to adenosine as an antagonist of antimalarials remains to be seen.

Summary. *Crithidia fasciculata* is competitively inhibited by several 2,4-diaminopyrimidine antagonists of folic and folinic acids. These *in vitro* inhibition patterns obtained in a chemically defined medium parallel the order of relative drug activities obtained in other laboratories on animal infections by any of several species of plasmodia. Poor correspondence exists, however, between bacterial inhibitions *in vitro* and inhibitions of plasmodia *in vivo* by these pyrimidines. The results suggest that this flagellate may be preferable to bacteria hitherto used for preliminary *in vitro* evaluation of antimalarials.

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1. Nathan, H. A., Cowperthwaite, J., and Hutner, S. H., *Bact. Proc.*, 1953, p70, abstract.

2. Cowperthwaite, J., Weber, M. M., Packer, L., and Hutner, S. H., *Ann. N. Y. Acad. Sci.*, 1953, v56, 972.

3. Falco, E. A., Russell, P. B., and Hitchings, G. H., *J. Am. Chem. Soc.*, 1951, v73, 3753.

4. Russell, P. B., and Hitchings, G. H., *ibid.*, 1951, v73, 3763.

5. Clarke, D. A., Buckley, J. M., Sternberg, S. S., Stock, C. C., Rhoads, C. P., and Hitchings, G. H., *Cancer Research*, 1952, v12, 255.

6. Falco, E. A., Goodwin, L. G., Hitchings, G. H., Rollo, I. M., and Russell, P. B., *Brit. J. Pharmacol.*, 1951, v6, 185.

7. Hitchings, G. H., Falco, E. A., Vanderwerff, H., Russell, P. B., and Elion, G. B., *J. Biol. Chem*, 1952, v199, 43.

8. Moulder, J. W., and Evans, Jr., E. A., *ibid.*, 1946, v164, 145.

9. Silverman, M., Ceithaml, J. J., Taliaferro, L. G., and Evans, Jr., E. A., *J. Infect. Dis.*, 1944, v75, 212.

10. McKee, R. W., in *Biochemistry and Physiology of Protozoa*, Academic Press, New York, 1952, p251.

11. Nathan, H. A., Cowperthwaite, J., and Carsted, K. W., unpublished.

12. Madinaveitia, J., and Raventós, J., *Brit. J. Pharmacol.*, 1949, v4, 81.

13. Felton, E. A., and Niven, Jr., C. F., *J. Bact.*, 1953, v65, 482.

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Tyrosinase in Frozen Sections and Fragments of Mouse Skin.*† (20804)

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The study reported here grew out of efforts to account for differences in results obtained by previous histochemical studies of enzymatic pigment-formation (dopa reactions) in normal mammalian skin(1,2) in contrast to more recent ones in which tyrosinase activity was demonstrated by various methods(3-6). It is important to account for these differences because of conflicting conclusions which could be drawn concerning the physiological genetics of mammalian coat-color pigmentation. For this reason it was considered necessary to attempt new histochemical demonstrations of tyrosinase activity in frozen sections and fragments of normal mammalian skin.

Materials and methods. The mouse genotype used was the triple homozygous recessive for pink eye, brown and maltese dilution (ppbbdd). Genes pp (pink eye) and brown (bb), either separately or in combination, do not cause any marked diminution in dopa oxidase activity although they do cause a drastic reduction in amount of hair pigmentation(7,8). The dilution factor (dd) reduces neither enzyme activity nor amount of pigment, but acts as a pigment clumping factor, resulting in a lighter appearing hair(7,8). Thus low background pigmentation and high enzyme activity should permit ready observation of newly formed pigment. The dorsal skins of 7-day-old mice were prepared by freezing and grinding as previously described (4), or sectioned at 30 μ on the freezing microtome. Incubation of skin samples was carried out at 38°C in 1½ or 2 ml of M/10 phosphate buffer, pH 6.8 (incubated controls), or in the same volume of buffer containing L-tyrosine at a concentration of 0.5 mg/

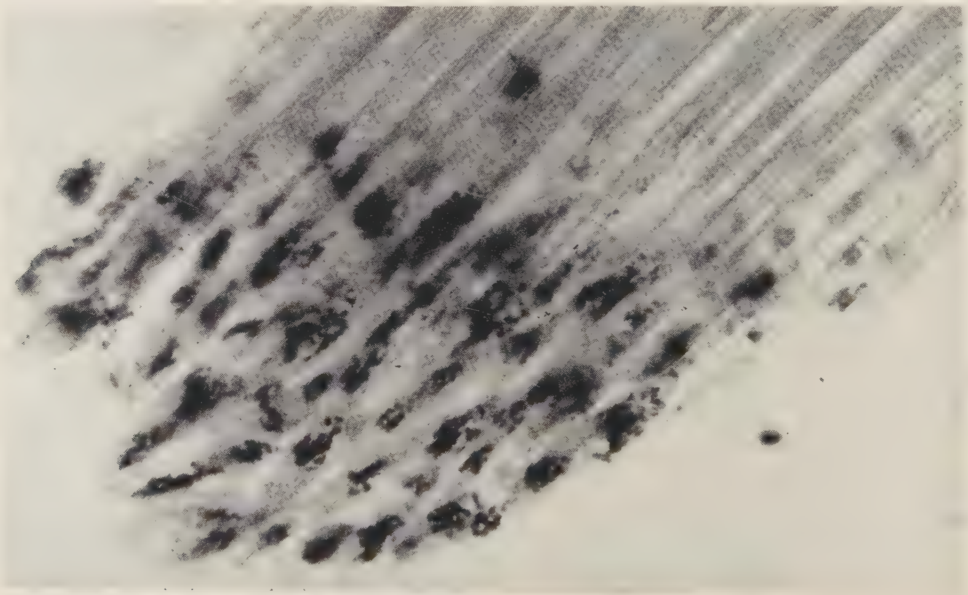

FIG. 1. Relatively discrete pigmentary details in hair bulbs of control skin fragment (incubated in buffer for 20 hr) (× 112).

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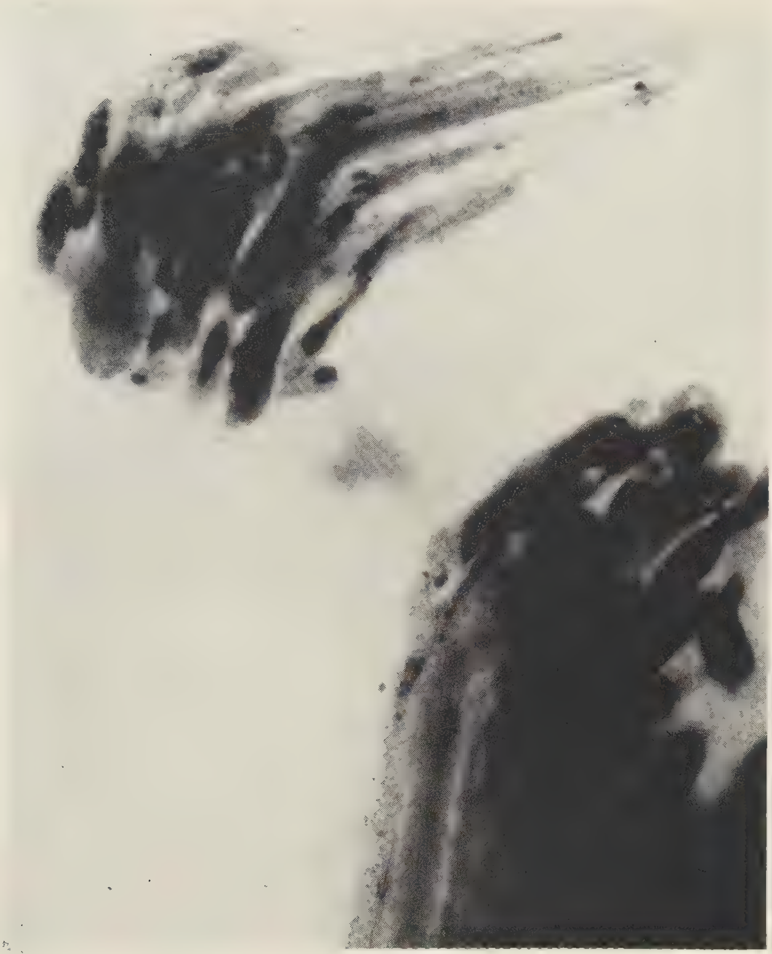

FIG. 2. Obliteration of discrete pigmentary details by newly formed pigment in hair bulbs of skin fragments incubated in tyrosine (20 hr) (× 112).

ml, in vessels of 5 ml capacity. The amount of skin incubated varied from a few fragments or sections to approximately ½ dorsal skin prepared as fragments or sections. As a check against the possibility that incubated control samples might undergo some darkening due to utilization of endogenous substrate, an additional control in the form of frozen, but unincubated, skin was prepared. After incubation, 3 hours in the case of the sections, 20 hours in the case of the fragments; the skin samples were fixed in 1:10 formol-alcohol (20 minutes), dehydrated in absolute alcohol (20 minutes), cleared in toluol (at least 20 minutes) and mounted in Permount (Fisher Scientific Co.).

Results. The darkening of both sections and fragments in tyrosine was apparent on gross examination. The tyrosine incubation media containing large amounts of skin developed a brown color. Even the skin samples incubated in buffer underwent a slight darkening as compared with the unincubated controls. Microscopic examination of hair bulbs under low power magnification revealed that the discrete pigmentary details, so readily apparent in the case of the controls (Fig. 1 and 3), tended to become obliterated when the skin was incubated in tysosine (Fig. 2 and 4). In these experimental cases many hair bulbs showed a dense oval mass of dark brown-black pigment, and when large amounts of skin were incubated, the peripheral portions of the hair bulbs, not normally

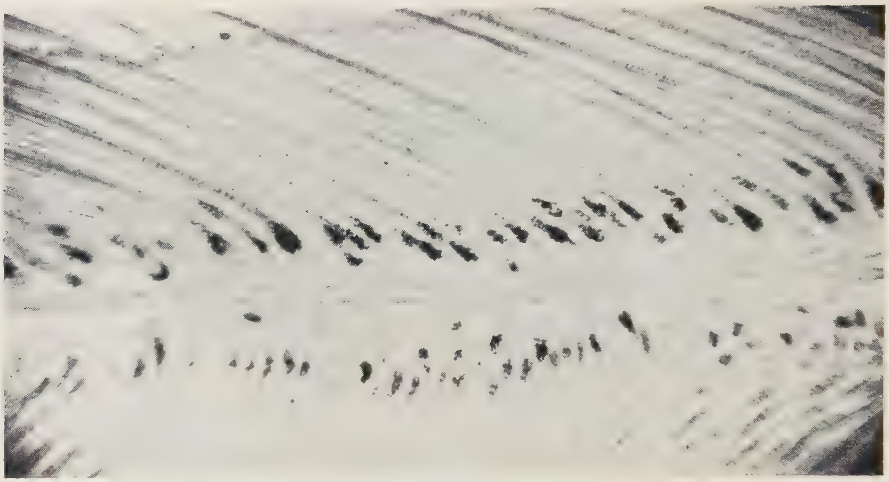

FIG. 3. Hair bulb pigmentary details in unincubated frozen skin sections. The skin was folded before sectioning so that 2 attached sections are shown (× 52).

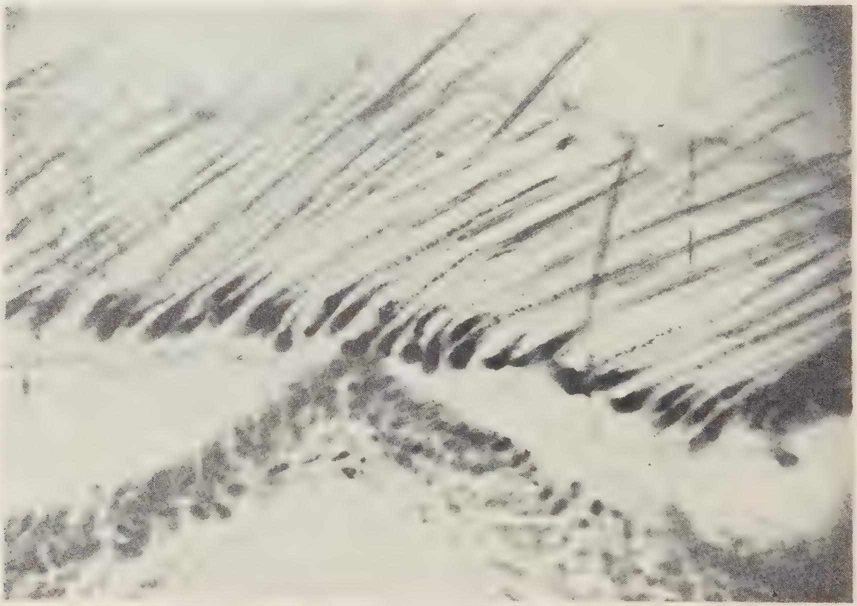

FIG. 4. Obliteration of pigmentary details by newly formed pigment in hair bulbs of frozen skin section (incubated in tyrosine for 3 hr) (× 52).

colored, took on the appearance of a brown region surrounding the brown-black central mass.

Although, on gross examination, skin incubated in tyrosine appears to undergo uniform darkening, microscopic examination reveals a good deal of variability. Thus an apparently uniform darkening is better described as a shift of the hair bulb population average in the direction of heavier pigmentation.

We also have observed that frozen sections of skin take up oxygen as well as do frozen fragments on incubation in tyrosine.

Discussion. The new demonstrations of tyrosinase activity reported here provide a basis for reconciling conflicting results and interpretations reported in the very recent and earlier studies cited above. Use can now be made of a variety of attacks for determining enzyme activity. Frozen sections are especially favorable for histological observation, while oxygen consumption measurements are independent of the amount of pigment already present in the hair bulbs at the beginning of an experiment. Independent checks on the enzymatic behavior of a given type of skin are therefore possible.

Summary. New histochemical demonstrations of tyrosinase activity in frozen sections and fragments of normal pigmented mouse skin are described. These demonstrations provide a basis for reconciling apparently conflicting results and interpretations arising from earlier studies, in which tyrosinase activity was not demonstrated, and later studies, in which this enzyme was readily demonstrated.

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1. Bloch, B., *Das Pigment.* In Jadassohn, J., *Handbuch der Haut-und Geschlechtskrankheiten,* 1927, v1, (1), 434.
2. Russell, W. L., *Genetics,* 1939, v24, 645.
3. Fitzpatrick, T. B., Becker, S. W., Lerner, A. B., and Montgomery, H., *Science,* 1950, v112, 223.
4. Foster, M., *J. Exp. Zool.,* 1951, v117, 211.
5. ———, *Genetics,* 1952a, v37, 581, (abstract).
6. ———, PROC. SOC. EXP. BIOL. AND MED., 1952b, v79, 713.
7. Russell, E. S., *Genetics,* 1948, v33, 228.
8. Russell, L. B., and Russell, W. L., *Genetics,* 1948, v33, 237.

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Interference Phenomenon of Browning and Gulbransen in Experimental Infection of Mice with *Trichomonas vaginalis.* (20805)

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The interference phenomenon of Browning and Gulbransen(1), *i.e.,* the antagonism of the trypanocidal dye stuff p-rosaniline hydrochloride toward 3,6-diaminoacridinium-10-methochloride (Acriflavine) has been primarily studied in trypanosomal infections with p-rosaniline resistant(1) and normally sensitive trypanosomes(2,3). According to Wright and Hirschfelder(4) the antagonism can also be demonstrated *in vitro* using the growth inhibiting activity of the dye stuffs toward yeast cells (*S. cerevisiae*). It could be expected that other organisms as long as they were sensitive to both the triphenylmethane and the acridine dye stuff would also show the interference. The pathogenicity of *Trichomonas vaginalis* for mice(5) and the response of this infection to local administration of p-rosaniline HCl and Acriflavine suggested an attempt to demonstrate the therapeutic antagonism of the 2 dye stuffs in the trichomonad infection.

Materials and methods. The strain of *T. vaginalis* was the same as that used in earlier experiments(5-7). Overnight growth in CPLM medium(8) was used in all instances. White mice from one colony were infected subcutaneously on the ventral side with 0.5 ml of a culture diluted to contain 500000 parasites as ascertained by actual count in a hemocytometer chamber. This dose represented at least 10 minimal infective doses. After 2 hours the marked site of the infection was injected locally with 1 ml of different dilutions of aqueous solutions of p-rosaniline hydrochloride and this was followed by a local injection of 1 ml of Acriflavine solutions at the same site after another interval of 2 hours. Control series with p-rosaniline alone and Acriflavine alone as well as groups of untreated mice were included in every experiment. Four days after the infection the mice were sacrificed, autopsied, and examined microscopically for presence or absence of active trichomonads.

Experimental. It had been found in preliminary experiments that p-rosaniline HCl and Acriflavine exerted a marked activity if administered locally under the experimental conditions described above. Acriflavine is one of the most active anti-trichomonad agents which is evident from the low CD_{50} of approximately 12 μg/ml. The corresponding CD_{50} of p-rosaniline HCl was 162 μg/ml.

TABLE I. Interference Phenomenon with p-rosaniline HCl and Acriflavine in the Subcutaneous Infection with *T. vaginalis*. Infection: 500000 parasites/mouse. Therapy: Local treatment with different doses of p-rosaniline and a constant dose (25 μg/ml) of Acriflavine.

p-rosaniline, μg/ml	Acriflavine, 25 μg/ml	No. of tests	No. of mice		% positive mice
			Neg	Pos	
100	—	6	0	48	100
100	+	6	5	43	89.6
50	+	5	6	28	82.4
25	+	4	11	17	60.7
12.5	+	4	8	24	75.0
6.25	+	2	14	2	12.5
3.125	+	2	15	1	6.25
—	+	8	53	7	11.7
—	—	8	0	56	100

The variation in this type of experiments was as a rule ±30%.

In the interference experiments inactive doses of p-rosaniline HCl, namely 100 μg/ml or less, were given while a constant active dose of Acriflavine, 25 μg/ml was used. The intervals of 2 and 4 hours after the infection were selected arbitrarily but are based on earlier experience in trypanosome experiments. The results of the experiments which are compiled from 2 to 8 tests with groups of 6 to 10 mice for the different dosages are given in Table I.

From the data in Table I it is evident that inactive doses of p-rosaniline hydrochloride markedly interfered with the effect of Acriflavine 25 μg/ml, a dose which otherwise controlled the trichomonad infection in about 90% of the cases. By the preceding administration of p-rosaniline in doses of 12.5 to 100 μg/ml the effect of Acriflavine was reduced to such a degree that dependent on the dose of p-rosaniline about 60 to 90% of the mice treated with the combination of dye stuffs showed the characteristic abscesses containing numerous active parasites. Smaller doses of 6.25 μg/ml or less did not antagonize the effect of Acriflavine. All untreated animals showed abscesses with numerous active parasites.

These observations seemed to indicate that the interference phenomenon of Browning and Gulbransen cannot only be demonstrated in trypanosome infections but equally well in the experimental trichomoniasis of mice. In one additional experiment the effect of graded doses of Acriflavine was titrated with and without previous administration of a constant inhibiting dose of p-rosaniline hydrochloride (50 μg/ml). In presence of the antagonist the CD$_{50}$ of Acriflavine was 35.5 μg/ml, whereas the CD$_{50}$ of Acriflavine alone was 8.8 μg/ml. No attempt has been made in the present study to analyze the mechanism of the interference. It seemed permissible to assume that in trichomonads as it was in the case of trypanosomes(9) and yeast cells(4) the adsorption of p-rosaniline by the parasite cell prevented the binding of Acriflavine which is essential for the anti-parasitic effect.

Summary. It was demonstrated that in experiments with mice infected with *T. vaginalis* a local treatment with inactive doses of p-rosaniline hydrochloride markedly antagonized an active dose of Acriflavine. The interference phenomenon of Browning and Gulbransen applies, therefore, also to *T. vaginalis*.

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1. Browning, C. H., and Gulbransen, R., *J. Pathol. and Bacteriol.*, 1922, v25, 395.

2. Schnitzer, R. J., *Z. f. Immunitaetsforsch.*, 1926, v47, 116.

3. Schnitzer, R. J., and Kelly, D. R., *J. Immunol.*, 1950, v64, 95.

4. Wright, H. N., and Hirschfelder, A. D., *J. Pharmacol. and Exp. Therap.*, 1930, v39, 39.

5. Schnitzer, R. J., Kelly, D. R., and Leiwant, B., *J. Parasitol.*, 1950, v36, 343.

6. Kelly, D. R., and Schnitzer, R. J., *J. Immunol.*, 1952, v69, 337.

7. Schnitzer, R. J., and Kelly, D. R., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 404.

8. Johnson, G., and Trussell, R. E., *ibid.*, 1943, v54, 245.

9. Hassko, A., *Z. f. d. ges. exp. Med.*, 1933, v87, 567.

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Urinary Excretion of Corticosteroids and Neutral 17-Ketosteroids in Adult Female Dogs. (20806)

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Chemical assays on urinary excretion of 17-ketosteroids in the dog are few(1-3) and studies of corticosteroid output by dogs are virtually nonexistant(4). In an investigation in which the crude urinary extract was fractionated with Girard's reagent-T, Glenn and Heftmann(3) found that normal dogs of either sex excreted less than 1 mg of neutral 17-ketosteroids per day. They found no increase in excretion of these compounds following administration of ACTH. No figures on the 24 hour excretion of corticosteroids in the dog have been published, to the authors' knowledge.

The present study was undertaken to establish more clearly the normal range of steroid excretion by female dogs and the extent to which this excretion was modified by factors generally recognized to influence adrenal hormone production or metabolism.

Method. Healthy adult female dogs were maintained on a stock diet, kept in metabolic cages and their urine collected using thymol as a preservative. All animals were catheterized at the beginning and end of each 24 hour period. The fresh urine was adjusted to pH 1 with concentrated HCl, an aliquot placed in a Hershberg-Wolfe extractor(5), and extracted continuously with redistilled methylene chloride for a period of 20 hours to obtain maximum yield(6). The methylene chloride containing the urinary extract was evaporated to about 50 ml, quantitatively transferred to a volumetric flask, and diluted to 100 ml. Twenty-five ml of the extract were transferred to a separatory funnel and 100 ml of redistilled ether added. Subsequent purification was essentially that of Heard, Sobel, and Venning(4). After washing with alkali and water, the purified extract was evaporated to dryness and taken up in methanol. Aliquots of this solution were used for corticoid and 17-ketosteroid estimation. Utilizing blue tetrazolium, estimation of reducing corticosteroids was carried out as previously described (7) with one simplification: acid alcohol (80 ml of methanol and 20 ml of concentrated HCl) was used instead of acid pyridine. The color of the reduced blue tetrazolium was not as long lasting as with pyridine but was stable for about one hour. The Zimmerman reaction with m-dinitrobenzene was used on extracts treated with Girard's reagent-T for neutral 17-ketosteroid assay(8).

Results, normal values. Significant amounts of neutral 17-ketosteroids are excreted by normal adult female dogs (Table I); the values are considerably lower than for adult human subjects(9-12). However, if expressed in terms of body weight, the daily excretion

TABLE I. Urinary Excretion of Neutral 17-Ketosteroids and Reducing Corticosteroids by Normal Female Dogs.

	17-ketosteroid excretion		Reducing corticosteroid excretion	
Dog wt	No. of specimens*	Mean Stand. dev.	No. of specimens*	Mean Stand. dev.
21	8	2.4 ± .80	11	1.3 ± .30
13	6	1.6 ± .63	4	1.3 ± .30
18	15	2.7 ±1.23	18	1.6 ± .62
14			12	2.3 ± .63
			4	2.0 ± .45
16.5	9	1.2 ± .42		
17.5	10	2.7 ± .59	14	1.8 ± .49
18	14	2.2 ± .68	28	2.0 ± .90
Avg		2.1 ± .73		1.8 ± .53

* Excretion in 24 hr urine specimens expressed in mg.

TABLE II. Effect of I.M. and I.V. ACTH on Urinary Excretion of Neutral 17-Ketosteroids and Corticosteroids by Normal Female Dogs.

		17-ketosteroids, mg/24 hr		Corticosteroids, mg/24 hr	
Dog No.	Total dose, mg/24 hr	Pretreatment excr.	Excr. during treatment	Pretreatment excr.	Excr. during treatment
1*	90	2.6	2.3	—	—
490	65	1.2	1.6	—	—
492	70	3.1	3.3	1.8	1.3
496	75	2.5	1.9	—	—
1†	60	—	—	2.4	4.0
45	40	—	—	2.7	2.8
1‡	25	2.2	4.0	2.2	6.3
10	23	—	—	1.1	2.2
	20	—	—	—	3.6
23	50	3.0	3.6	2.0	3.4
	30	—	4.3	—	5.6
45	25	—	—	1.3	4.0
	25	—	—	2.6	6.0
	25	—	—	—	5.5
496	20	3.5	—	2.6	3.3
	20	—	5.0	—	4.8
	25	—	5.0	—	6.1

* Intramusc. inj. every 24 hr. † Continuous 24 hr intramusc. drip. ‡ Continuous 24 hr intrav. drip.

TABLE III. Effect of a Single Injection of Cortisone on Urinary Excretion of Neutral 17-Ketosteroids and Corticosteroids by Normal Female Dogs.

		17-ketosteroids, mg/24 hr		Corticosteroids, mg/24 hr	
Dog No.	Total dose, mg	Pretreatment excr.	Excr. following inj.	Pretreatment excr.	Excr. following inj.
23*	200	3.0	5.5	1.8	4.2
	200	—	—	1.8	4.2
		—	—	—	2.1‡
	200	—	—	2.7	5.9
		—	—	—	1.8‡
496†	75	3.5	5.4	3.0	3.8
	100	—	—	2.3	2.8
	200	—	—	2.2	1.2

* Dog No. 23, intramusc. inj. † Dog No. 496, intrav. inj. ‡ Excretion during 2nd 24 hr following cortisone.

would be quite similar to that of the adult human female, although somewhat more variable. The average excretion of neutral 17-ketosteroids for the 6 adult female dogs was 2.1 mg/24 hr. The range of corticosteroid excretion was similar to that obtained by the same methods on adult human subjects(13). There was less daily variation than observed for ketosteroid excretion. The average excretion for blue tetrazolium reducing substances for seven dogs was 1.8 mg/24 hr (Table I).

Effect of ACTH administration. Intermittent intramuscular injections of ACTH caused no detectable increase in 17-ketosteroid output (Table II). Continuous intramuscular infusions resulted in some elevation in the urinary corticosteroid level in one out of 2 dogs. Continuous intravenous administration of ACTH, however, caused a consistent rise in 17-ketosteroid and corticosteroid excretion. The increase in 17-ketosteroid output was never greater than 180% of the pretreatment excretion, whereas corticosteroid output was increased from 210 to 330% following I.V. ACTH.

Effect of cortisone (Table III). Intramuscular and intravenous administration of cortisone was followed by some increase in 17-

TABLE IV. Hepatic Bloodflow and Reducing Corticoid Excretion in the Dog.

Dog No.	Procedure	Time, P.O. days	Corticoid excretion, mg/24 hr	R.B. dye clearance[‖]
45	Normal	—	2.6 (9)[*]	102
	Eck fistula	5– 40	3.0 (3)	41
		41– 77	1.1 (9)	38
		93–128	.5 (5)	39
	Control	63	.8 (1)	
	I.V. ACTH[†]	63– 99	3.4 (4)	
	Control	129	.5 (1)	
	I.V. ACTH[‡]	129–130	.65 (2)	
	Control	76	1.8 (1)	
	I.M. cortisone[§]	77	2.8 (1)	
496	Normal	—	2.1 (13)	105
	Reverse Eck	8– 71	.91 (14)	85

[*] ( ) No. of determinations from which avg value was obtained.

[†] 16 mg/24 hr (Armour preparation).

[‡] 45 mg/24 hr.

[§] 335 mg, I.M.

[‖] Rose Bengal dye clearance(17).

ketosteroid excretion in the 2 dogs studied. The effects of I.M. cortisone injection on corticosteroid output were irregular in one animal while I.V. administration of this hormone had no effect on corticosteroid excretion in a second animal.

Effect of operative procedures on corticoid excretion. Bloodflow through the liver was reduced and increased by Eck and reverse Eck fistula formation respectively. A reduced bloodflow through the liver did not significantly alter corticoid excretion for the first 40 days in the animal studied (Table IV). However, as the animal became debilitated, the corticoid excretion declined. The response to ACTH was similar to that of a normal animal until the health of the animal declined, at which time there was essentially no response to ACTH even though administered in larger doses than previously. Formation of a reverse Eck fistula was associated with a reduced corticoid excretion which remained at approximately half the normal value during the 71 days of observation. This animal remained in good health throughout the period of observation.

A few other miscellaneous observations were made on corticoid excretion in the dog. One dog that normally excreted an average of 1.3 mg of corticoids daily excreted 0.3 mg after adrenalectomy while receiving 1 mg of DOCA daily. One hypophysectomized male dog excreted 0.65 mg of corticoids daily one year after hypophysectomy. Common bile duct obstruction and diverting bile excretion into the urine did not significantly alter the 24 hour excretion of corticoids in the urine of one dog. A comparison of corticoid excretion following intrasplenic and intramuscular injections of cortisone gave inconclusive results on one dog with an exteriorized spleen.

Discussion. Female dogs excrete a substantial amount of corticosteroids in their urine. Spontaneous daily variation is small enough so that significant increases or decreases in corticosteroid output may be recognized with reasonable accuracy.

The normal values for 24 hour excretion of 17-ketosteroids reported here are higher than those obtained by Glenn and Heftmann(3). This difference is most probably due to the fact that their extraction time was limited to 1.5 hours. Pincus and Romanoff(6) have shown that, so far as corticosteroids are concerned, 4 hours of extraction will result in a 35% yield of the potential maximum.

Intramuscular ACTH did not produce consistent elevations in 17-ketosteroid or corticosteroid output. However, intravenous ACTH caused increased excretion of both groups of steroids.

The relatively small increases in urinary reducing steroids following large doses of cortisone indicate that this substance is largely altered in the normal animal prior to excretion.

The effect of adrenalectomy on the excretion of tetrazolium reducing substances in urine suggests that most of the material included in this estimation was adrenal in origin. Similar results have been obtained on adrenal insufficient human subjects using the same method(13). The urine of dogs contains vit. A in quantities up to 450 I.U. per 100 ml(14) which reduces phosphomolybdic acid(15). Investigation has shown that with the present method of corticosteroid estimation, errors due to vit. A reduction will be less than 2%.

Alteration of the blood supply to the liver and splenic injections of cortisone give no clear evidence concerning the role of the liver in the metabolism of corticosteroids(16) although the reduced excretion following reverse

Eck fistula in one animal suggests that the liver must play a part in the degradation of these hormones. No evidence was obtained that reducing steroids are excreted in the dog's bile.

The reduced excretion of corticosteroids that eventually occurs in the Eck fistula animal can best be explained on the basis of debility and inanition which developed during the postoperative period of study.

Summary. Normal values for neutral 17-ketosteroids and reducing corticoids have been presented for adult female mongrel dogs. Stimulation of the adrenal glands of the dog by continuous intravenous infusion of ACTH increased both the reducing corticoid and neutral 17-ketosteroid excretion. Additional observations are reported regarding factors that influence the excretion of reducing corticoids by the adult female dog.

1. Paschkis, K. E., Cantarow, A., Rakoff, A. E., Hansen, L. P., and Walkling, A. A., PROC. SOC. EXP. BIOL. AND MED., 1943, v53, 213.

2. Lawrence, G. H., *Endocrin.*, 1949, v45, 383.

3. Glenn, E. M., and Heftmann, E., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 147.

4. Heard, R. D. H., Sobel, H., and Venning, E. H., *J. Biol. Chem.*, 1946, v165, 699.

5. Hershberg, E. B., and Wolfe, J. K., *ibid.*, 1941, v141, 215.

6. Pincus, G., and Romanoff, L. P., *Fed. Proc.*, 1950, v9, 101.

7. Chen, C., and Tewell, H. E., Jr., *ibid.*, 1951, v10, 377.

8. Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, v32, 1312.

9. Mason, H. L., and Engstrom, W. W., *Physiol. Rev.*, 1950, v30, 321.

10. Venning, E. H., and Kazmin, V., *Endocrin.*, 1946, v39, 131.

11. Talbot, N. B., Albright, F., Saltzman, A. H., Zygmuntowicz, A., and Wixom, R., *J. Clin. Endocrin.*, 1947, v7, 331.

12. Corcoran, A. C., and Page, I. H., *J. Lab. Clin. Med.*, 1948, v33, 1326.

13. Chen, C., Wheeler, J., and Tewell, H. E., Jr., *ibid.*, 1953, v42, 758.

14. Lawrie, N. R., Moore, T., and Rajagopal, K. R., *Biochem. J.*, 1941, v35, 825.

15. Burstein, S., *Endocrin.*, 1952, v50, 412.

16. Nelson, D. H., and Harding, B., *Fed. Proc.*, 1952, v11, 379.

17. Hough, V. H., and Freeman, S., *Am. J. Physiol.*, 1942, v138, 184.

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Antibiotic Combinations and Resistance: Response of *E. coli* to Antibiotics, Singly and in Pairs.* (20807)

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In a previous study(1) it was shown that the development of resistance in strains of staphylococci and in a strain of enterococcus to penicillin (Pen), streptomycin (S) or erythromycin (E) *in vitro* could be depressed and delayed by repeated subcultures in the presence of combinations of E with either Pen or S. Further observations(2) with strains of staphylococci and certain streptococci repeatedly subcultured in Pen, S, E, oxytetracycline (Terramycin, "T") and coloramphenicol (C) and in 8 additional pairs of these antibiotics confirmed these findings and indicated further that: 1) the rate and degree of resistance which developed against individual antibiotics to which the organisms were exposed varied with the antibiotics and sometimes with the strain of organism; 2) subcultures on the pairs of antibiotics resulted in increased resistance to the homologous mixture, but usually of lower degree than against the individual components when used separately; 3) when increases in resistance to the

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TABLE I. Sensitivity of 6 Parent Strains of *E. coli* to Antibiotics Singly and in Certain Pairs.

Minimum inhibiting concentration, $\mu g/ml$														
S	C	T	P	S+C	S+T	C+T	S+P	C+P	T+P	A	N	Pen	B	E
													×1000	
6.3	6.3	6.3	1.6	3.1+3.1	6.3+3.1	3.1+1.6	6.3+.8	6.3+.8	6.3+1.6	12.5	3.1	200	15	100
6.3	25	"	1.6	6.3+6.3	"	6.3+3.1	"	"	"	"	12.5	"	"	200
6.3	6.3	"	.8	"	"	3.1+1.6	"	3.1+.4	3.1+ .8	"	"	400	"	"
6.3	12.5	"	.8	"	"	"	"	"	6.3+1.6	25	6.3	200	"	"
12.5	6.3	"	3.1	"	"	"	"	6.3+.8	"	"	"	"	"	"
6.3	25	"	1.6	"	"	6.3+3.1	"	"	"	"	"	"	"	"

S = streptomycin; C = chloramphenicol; T = Terramycin (oxytetracycline); P = polymyxin B; A = Aureomycin (chlortetracycline); N = neomycin; Pen = penicillin; B = bacitracin; E = erythromycin.

homologous individual components differed appreciably, the increase in resistance which developed against the pair corresponded more closely to that of the antibiotic which alone produced the smaller change; and 4) increases in resistance to the individual components of a mixture resulted from repeated exposures to the combination, but these increases were generally of smaller magnitude than those which developed against the same antibiotics when used separately. The present report deals with further extensions of these observations to strains of *Escherichia coli* using S, T, C and polymyxin B (Aerosporin, "P") separately and in mixtures of all 6 possible pairs.

Materials and methods. The 6 strains of *E. coli* used in this study were recently isolated from the blood or urine of patients and identified by Marion E. Lamb of the Mallory Institute of Pathology. The polymyxin B (Aerosporin) was furnished by Dr. Donald S. Searle of Burroughs Wellcome & Co.; the other antibiotics were similar to those employed in the previous studies(1,2). Heart infusion agar (Difco), pH 7.2±, was used with and without the incorporation of serial 2-fold concentrations of the antibiotics, singly and in combinations; pairs of antibiotics were always used in a constant ratio for each pair, the proportions being determined by the relative sensitivity of the parent strains to the respective antibiotics. The serial subcultures and tests for sensitivity and cross-resistance were carried out as in previous studies(1-3).

Results. Resistance to homologous antibiotics. The sensitivity of the 6 parent strains of *E. coli* to all of the antibiotics used in this study are listed in Table I. It is seen that the antibiotics used in the various combinations exerted no important synergistic or antagonistic effects on the parent strains. In general the minimum inhibiting concentration (M.I.C) of each of the antibiotics in the combinations was the same as that of the component which was most active, except for mixtures C + T and C + P which appeared to be approximately additive, *i.e.*, about ½ the M.I.C. of each of the components was required when they were used together. The changes in the M.I.C. of the homologous antibiotics resulting from 20 successive subcultures on agar containing S, C, T or P, individually or in pairs, are depicted in Fig. 1 and 2. There were some individual variations among the strains in the rate and degree to which resistance increased. Marked and rapid increases in resistance occurred regularly with S and C; increases in resistance to T also occurred in all strains but these were of smaller magnitude, while little or no increases in resistance to P occurred, except in strain 2. The increases in resistance to homologous pairs of antibiotics were most marked with C + T and only slightly less with S + C; only small increases in resistance or none at all resulted from the subcultures in each of the other 4 combinations.

Resistance and cross-resistance to individual antibiotics. The changes in resistance to individual antibiotics that resulted from the 20 subcultures of each of the 6 strains of *E. coli* in S, C, T or P, separately and in pairs, are listed in Table II. No significant changes in sensitivity or resistance to Pen, bacitracin (B), or E resulted from these subcultures; the results of the tests with these antibiotics are, therefore, not listed in this table. The exposures to S alone resulted in slight increases in sensitivity of most of the strains to C, T and chlortetracycline (Aureomycin,

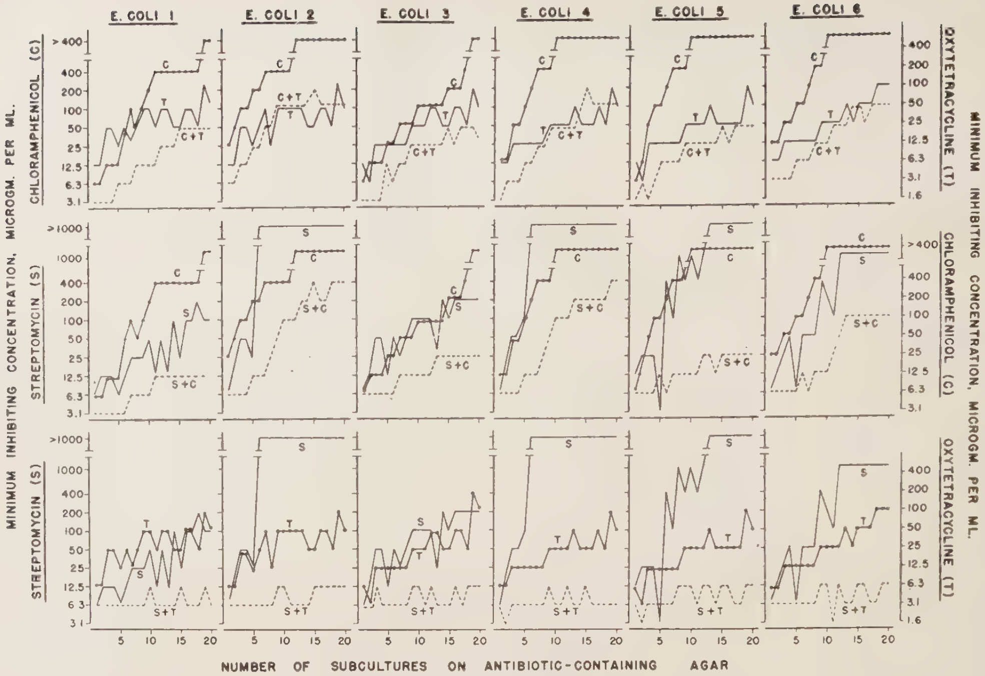

FIG. 1. Resistance developing during successive subcultures on streptomycin, chloramphenicol or oxytetracycline, alone and in pairs.

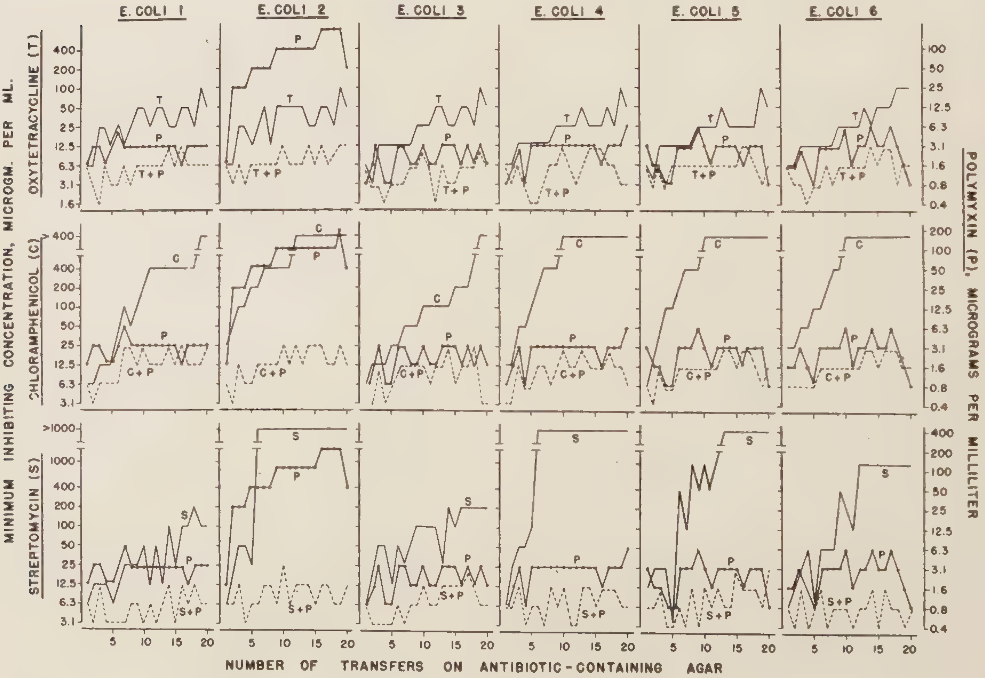

FIG. 2. Resistance to polymyxin combined with streptomycin, chloramphenicol, or oxytetracycline and to each antibiotic individually.

TABLE II. Changes in Sensitivity of *E. coli* after Subcultures on Antibiotic-Containing Media.*

Antibiotic used in tests†	*E. coli,* strain No.	Antibiotic (s) on which strain was subcultured 20 times									
		S	C	T	P	S+C	S+T	C+T	S+P	C+P	T+P
S	1	16	½	1	1	4	800	1	2	1	1
	2	800	¼	¼	2	800	800	½	40	½	½
	3	32	1	½	1	4	16	½	8	1	1
	4	800	½	½	1	800	8	1	8	½	1
	5	800	¼	½	1	16	160	1	32	½	½
	6	80	½	1	½	800	8	1	2	½	½
C	1	½	128	4	1	8	1	128	1	2	8
	2	¼	64	4	¼	64	2	64	½	4	2
	3	½	64	2	½	4	1	64	1	4	1
	4	¼	64	8	1	64	1	64	1	4	8
	5	½	64	2	1	8	4	64	½	8	4
	6	½	64	8	½	32	2	64	1	2	2
T	1	¼	32	16	1	1	2	16	¼	4	2
	2	¼	8	8	1	2	1	32	¼	4	2
	3	½	2	8	½	1	1	8	½	2	2
	4	⅛	4	8	1	2	1	32	½	1	2
	5	½	8	8	1	2	1	8	¼	1	2
	6	¼	4	16	1	2	2	32	1	1	2
P	1	1	¼	1	2	1	1	1	½	4	1
	2	1	¼	1	32	2	1	2	1	1	8
	3	1	½	1	1	1	1	½	1	2	1
	4	2	2	1	4	2	1	2	2	8	1
	5	1	½	½	1	2	1	2	1	1	1
	6	½	½	1	2	1	1	1	1	½	2
A	1	1	64	8	1	4	4	16	½	2	2
	2	¼	8	8	1	4	2	16	½	8	2
	3	¼	2	8	1	1	2	8	½	2	2
	4	½	8	4	2	1	1	8	1	4	2
	5	¼	32	4	1	1	1	4	1	2	2
	6	¼	8	16	1	2	2	16	1	4	4
N	1	32	¼	1	1	1	4	½	4	1	1
	2	2	⅛	¼	2	2	8	1	½	1	1
	3	32	1	¼	½	8	16	1	2	½	1
	4	2	¼	1	1	2	4	1	2	1	1
	5	1	¼	1	1	8	4	1	2	1	1
	6	32	¼	½	2	2	8	2	4	1	1

* Expressed as quotient derived by dividing minimum inhibiting concentration (M.I.C.) of strain after 20 subcultures in the antibiotic by the M.I.C. of the same strain not previously exposed to antibiotic, when tested at the same time.

† S = streptomycin; C = chloramphenicol; T = Terramycin (oxytetracycline); P = polymyxin B; A = Aureomycin (chlortetracycline); N = neomycin.

"A"), no change in their sensitivity to P and cross-resistance of considerable degree in ½ of the strains to neomycin (N). The increase in resistance to C resulting from the exposures to that antibiotic was accompanied by cross-resistance of varying degrees to T and A and by some increases in sensitivity to S, P and N. The subcultures in T produced considerable cross-resistance to C and A, but no changes (or slightly increased sensitivity) to S, P or N. Repeated exposures to P did not affect the sensitivity to any of the other antibiotics. The effects of the subcultures in the pairs of antibiotics on the sensitivity of the strains to individual antibiotics varied considerably. The 3 combinations containing S all produced moderate or marked increases in resistance to that antibiotic; the combination of S with C may have reduced but did not prevent the development of resistance to the latter, but resistance to T or to P did not result from the exposures to each of these antibiotics in combination with S. Cross-resistance to N resulted irregularly from subcultures in each of the 3 combinations containing S, but the least change occurred when

TABLE III. Changes in Sensitivity to Pairs of Antibiotics Resulting from Subcultures in Both or Each.

Antibiotic (s) to which strain was exposed	Antibiotics with which strain was tested	Changes in M.I.C.* *E. coli*, strain No. 1	2	3	4	5	6
S	S+C	4	1	½	2	1	½
C	"	2	1	½	2	½	1
S+C	"	4	64	4	64	4	16
S	S+T	4	1	1	½	1	1
T	"	4	2	2	2	2	1
S+T	"	4	4	4	1	4	4
C	C+T	32	8	2	32	16	8
T	"	4	8	8	16	4	16
C+T	"	16	16	8	32	16	16
S	S+P	1	1	1	1	1	½
P	"	1	8	1	1	1	½
S+P	"	1	2	1	1	4	2
C	C+P	4	1	1	2	1	2
P	"	1	1	2	2	1	2
C+P	"	4	2	1	1	2	2
T	T+P	½	1	1	2	1	½
P	"	½	2	1	1	1	1
T+P	"	2	4	2	1	2	2

* See footnotes to Table II.

S was paired with P; some increase in sensitivity to T resulted from subcultures in the latter mixture. The combination C + T failed to prevent or depress the development of resistance to either of these agents. The strains exposed to C + P showed slight and irregular increases in resistance to each of these agents separately and also to T and A but no effect on the sensitivity to any of the other antibiotics. Subcultures in T + P resulted in only minor and irregular increases in resistance to these agents separately and also some cross-resistance to C and A.

Resistance to pairs of antibiotics. The changes in resistance to pairs of antibiotics which resulted from the 20 transfers in the homologous mixtures and in their respective components are listed in Table III. With minor exceptions, the sensitivity of each strain to the pairs of antibiotics was not altered by the exposures to the single constituents of the mixtures except in the case of the strains which had been subcultured in either C or T; almost all of these strains increased in resistance to the mixture of these 2 antibiotics.

Effect of antibiotic exposures on morphologic and biochemical characteristics. All of the strains which had been subcultured on agar containing antibiotics, singly or in pairs, were transferred 1 or 2 times on antibiotic-free broth and then studied simultaneously with their respective parent strains which had not been exposed to antibiotics. No changes were made out in the appearance of the different strains in gram-stained preparations,

TABLE IV. Summary: Resistance of *E. coli* Resulting from 20 Subcultures on Antibiotics, Singly and in Pairs.*

Tested with†	Subcultured 20 times in: S	C	T	P	S+C	S+T	C+T	S+P	C+P	T+P
S	++	0,—	0,—	0	+,++	+,++	0	+,++	0	0
C	0,—	++	+	0	+,++	0,+	++	0	+	0,+
T	—	+,++	+,++	0	0	0	+,++	0,—	0,+	0
P	0	0,—	0	0,++	0	0	0	0	0,+	0,+
S+C	0,+	0			+,++					
S+T	0,+		0,+			+				
C+T		+,++	+,++				++			
S+P	0			0,+				0,+		
C+P		0,+		0					0,+	
T+P			0	0						0,+
A	0,—	+,++	+,++	0	0,+	0,+	+,++	0	0,+	0,+
N	0,++	0,—	0,—	0	0,+	+	0	0,+	0	0
Pen B E	0	0	0	0	0	0	0	0	0	0

* Changes in resistance as compared with inhibiting concentration of parent strain not previously exposed to antibiotic and tested simultaneously; these changes are expressed as follows: 0 = no significant change, *i.e.* quotient described in footnote to Table II is ½, 1 or 2; + = 4- or 8-fold increase in resistance; ++ = 16-fold or greater increase in resistance; — = more sensitive, *i.e.* requiring ¼ or less of original inhibiting concentration.

† See footnote Table I.

nor in the appearance of the colonies which they formed on heart-infusion agar and on eosin-methylene-blue agar. All of the strains failed to utilize citrate, and showed negative Voges-Proskauer and positive methyl-red tests. Tests for penicillinase production were done by a method previously employed with *Staph. aureus*(4). *E. coli* strains 2 and 3 produced penicillinase in these tests while the other 4 did not; this was true of both the parent strains and those which had been subcultured in the antibiotics singly or in pairs. In order to visualize more clearly the changes that have been described, the results shown in Tables II and III are summarized in Table IV.

Summary and conclusions. The data presented confirm and extend the observations previously made with staphylococci and some streptococci using other antibiotic combinations. For *E. coli,* and within the limitations of the methods employed, it was shown that following repeated subcultures in pairs of antibiotics, resistance generally developed more slowly and to a smaller degree than when the same organisms were exposed to the same antibiotics individually. There were wide variations, however, depending mainly on the antibiotics used, but perhaps also with the strain of organism. Cross-resistance to neomycin frequently accompanied increases in resistance to streptomycin resulting from exposure to the latter, alone or in combinations. Likewise, cross-resistance to oxytetracycline and chlortetracycline from exposures to chloramphenicol and to chloramphenicol and chlortetracycline following subcultures on oxytetracycline accompanied the development of resistance to the homologous antibiotic, whether present alone or paired with another agent.

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1. Purcell, E. M., Wright, S. S., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 124.

2. Wright, S. S., *et al., J. Lab. and Clin. Med.,* 1953, v42, 877.

3. Gocke, T. M., and Finland, M., *ibid.,* 1951, v38, 719.

4. Haight, T. H., and Finland, M., *Am. J. Clin. Path.,* 1952, v22, 806.

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Amyloidosis and Renal Lesion Induced in Mice by Injection with Freund-Type of Adjuvant.* (20808)

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During attempts to produce injury to collagen in the mouse by subcutaneous injections of homologous collagen incorporated in the Freund-type of adjuvant, it was noted that deposits of amyloid occurred in certain tissues of the animals. Subsequent studies revealed that collagen played no role in the induction of amyloidosis and that the injury was due to the adjuvant. The Freund-type of adjuvant has been shown to enhance in the animal host circulating antibody formation against a variety of antigens(1) and to potentiate the production of allergic encephalitis (2). Another reaction to this adjuvant has been the development of amyloidosis. Although numerous reports have appeared in which this type of adjuvant has been used in many animal species, amyloidosis has been reported only in the duck following injection of killed malarial parasites combined with paraffin oil and killed tubercle bacilli(3). Experimental amyloidosis has been induced in several species of animals with diverse materials but only after long periods of treatment and with highly variable incidence. The observation that repeated injections of adjuvant induce amyloid in the mouse may offer a more efficient method of inducing amyloid-

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osis for the study of the pathogenesis of this disease in the experimental animal.

Materials and methods. W-Swiss, H-line mice[†] of both sexes 6 to 9 weeks old, weighing 15 to 20 g were employed. Their diet was made up of "Big Dog" pellets, water, bread and milk. The adjuvant was prepared as previously described(4), and consisted of a water-in-oil emulsion in which killed microorganisms were suspended. The oil phase consisted of an autoclaved mixture of 1.5 cc of Arlacel A[‡] (Mannide monoleate), an emulsifying agent, and 8.5 cc of Bayol F,[§] a paraffin oil of low viscosity; in this mixture were suspended 8 mg of killed and dried *M. butyricum*, or *Nocardia asteroides*,[‖] or Group A streptococci,[¶] type 6, strain S43/101/7. The aqueous phase consisted of 30 cc of 1-10000 dilution of acetic acid[**] in distilled water. As the latter was added by drops to the oil phase it was emulsified by repeatedly filling and emptying a syringe in the mixture, no needle was attached to the syringe. The emulsion was stored for several weeks at 4°C without evident separation. The mice were injected subcutaneously or intramuscularly at 7-day intervals. Untreated paired litter mates sacrificed simultaneously with the experimental animals served as controls. The tissues were fixed in 10% neutral formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin, congo red, and, in some instances, with crystal violet for metachromasia. Specimens of liver and spleen were treated with Lugol's solution and sulfuric acid for amyloid.

Pathologic findings. No deaths occurred during the experimental period. All animals were sacrificed 7 days after the last injection. The spleen and liver were usually found to be greatly enlarged, and in many instances the kidneys appeared large, pale and waxy. Gross specimens of spleen, liver, and kidney failed to give the characteristic stain for amyloid with Lugol's solution and sulfuric acid. The *spleen* was 3 to 4 times normal in size and firm with a tense capsule. The Malpighian corpuscles were grossly visible and the cut surface had a glassy appearance. Microscopic examination showed the loss of normal architecture and a homogeneous acidophilic deposit surrounding the corpuscles. This material was also found in the walls of the central arterioles, beneath the capillary endothelium of the follicles and walls of the vascular sinuses (Fig. 1). These deposits showed the characteristic congo red stain for amyloid. The red color was less intense than in human material employed as a control. The crystal violet stain for metachromasia was characteristic: the amyloid stained red-violet and tissue blue. The *livers* of the treated mice were enlarged, firm, pale and the cut surface appeared glassy. The amount of amyloid was less than in the spleen. It was most extensive in the sinusoids of the lobules between the endothelial and liver cells, resulting in pressure atrophy of the latter. Periportal round cell infiltration was also prominent in many of the sections. Amyloid was also found in the hepatic arteries and branches of the portal veins (Fig. 2). The involved *kidneys* were enlarged, pale, waxy, and almost bloodless. On cut section, the corticomedullary junction was lost. The capsules were not adherent and the surface was smooth. Microscopically, the striking features were the marked dilation of the tubules, often appearing as multiple cystic areas and the dilation of Bowman's space of the glomeruli (Fig. 3). The tubular epithelium was atrophied and the tubules contained colloid casts but no inflammatory cells. The glomerular capillary loops were ischemic and larger arteries were free of blood. Amyloid deposits were found in the medulla near the papilla where it occurred in the capillary walls beneath the endothelium. The tip of the

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[†] Obtained from the Rockefeller Institute for Medical Research.

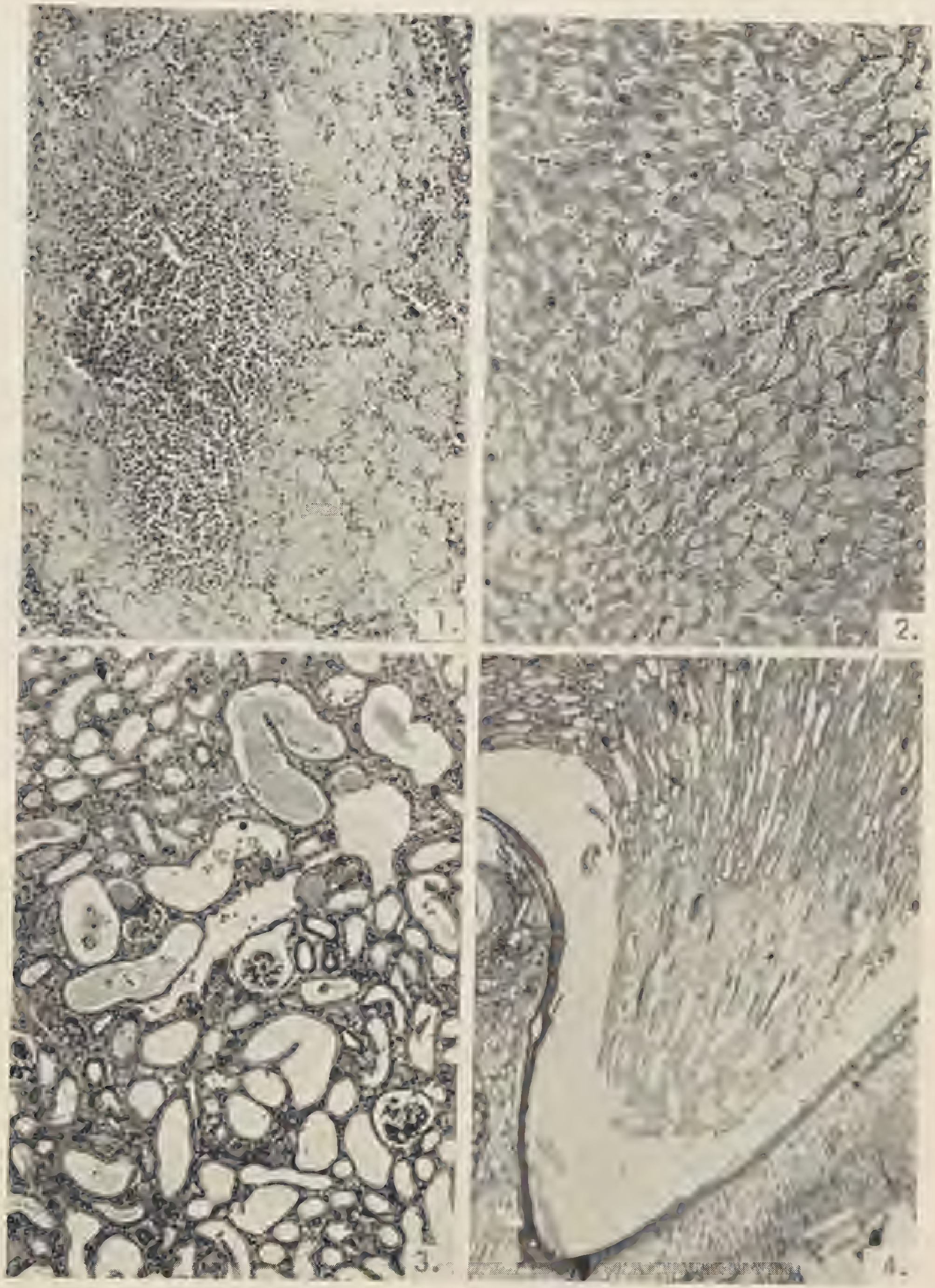
[‡] Distributed by the Atlas Powder Co., Wilmington, Del.

[§] Distributed by the Stanco Distributors, New York, N. Y.

[‖] *M. butyricum* and *Nocardia asteroides* were kindly supplied by Dr. Jules Freund of Public Health Research Institute, City of New York.

[¶] Group A streptococci kindly supplied by Dr. R. C. Lancefield of the Hospital of the Rockefeller Institute.

[**] Acetic acid was used as a solvent for the collagen, but it is not essential.



Microscopic appearance of mouse tissues after 10 weekly injections of Freund-type adjuvant.

FIG. 1. Section of spleen showing perifollicular infiltration of amyloid. × 90.

FIG. 2. Section of liver with moderate infiltration of amyloid and compression atrophy of hepatic cells. × 90.

FIG. 3. Section of kidney showing marked dilation of tubules, atrophy of tubular epithelium, colloid-like casts, and some interstitial fibrosis; glomeruli show little blood, small deposits of amyloid, and dilation of Bowman's space. × 100.

FIG. 4. Section of kidney showing necrosis of tip of papilla with deposits of amyloid lining capillary walls. × 75.

papilla was necrotic (Fig. 4). The glomeruli were only slightly involved by amyloid but patches of it were demonstrated by crystal violet staining. The interstitial tissue showed no evidence of acute inflammatory reaction but small areas of fibrosis were found in some instances. No obstruction was found in the ureters.

The injection sites did not ulcerate and appeared as encapsulated areas containing material similar to that injected which on culture revealed no growth. In those animals showing amyloid, the spleen was invariably involved; the liver less frequently; and the kidney lesion was never observed unless there was involvement of both liver and spleen. Amyloid was not found in other organs. Although studies were not made for plasma cells in all tissues, no foci of these cells were found in bone marrow sections. In 40 control litter mates neither amyloid nor renal lesions were found, nor have these lesions occurred spontaneously in older mice of the W-Swiss H-line as observed in Strain A mice by Gorer (5) and by Dunn(6).

Production of amyloid by various components of the Freund adjuvant. Since the Freund-type of adjuvant consists of 3 different components, an oil, emulsifying agent, and killed mycobacteria, it seemed of interest to determine which of these components were essential for the production of the amyloid. A group of 30 mice was injected with 0.2 cc of whole adjuvant every 7 days for 10 injections and killed 7 days after the final injection. Of this group 29 mice (Table I) showed amyloidosis; the spleen was involved in all and the liver in 16; the renal lesion occurred in 16. When the emulsifying agent, Arlacel A, and the paraffin oil, Bayol F, were employed in 10 mice under similar conditions no amyloid deposits or renal lesions were seen. This finding suggested that the killed *M. butyricum* were essential. However, *M. butyricum* alone also failed to induce amyloidosis. When *M. butyricum* was suspended in either Arlacel A or Bayol F, amyloid and the renal lesion did appear but the incidence was less than when the complete adjuvant was employed (Table I). It seemed desirable to determine whether or not some other microbial agent which had acid fast properties similar to the mycobacteria might be effective in inducing amyloidosis. *Nocardia asteroides* was selected because this organism has been shown to potentiate sensitization(7). Of 20 mice treated 13 showed amyloid of the spleen and liver; and 4 the renal lesion (Table I).

TABLE I. Incidence of Amyloidosis and Renal Lesion in Mice Injected with Components of Freund-Type Adjuvant.

		No. of mice showing		
		Amyloid		
Material inj.	No. of mice used	Spleen	Liver	Renal lesion
Arlacel, Bayol, & *M. butyricum*	30	29	16	16
Arlacel & Bayol	10	0	0	0
M. butyricum	10	0	0	0
Arlacel & *M. butyricum*	10	6	6	1
Bayol & *M. butyricum*	10	5	5	2
Arlacel, Bayol & *N. asteroides*	20	13	13	4
Arlacel, Bayol & Group A streptococci, Type 6, Strain S43/101/7	20	3	0	0

It was also of interest to determine whether bacteria without acid fast properties, such as Group A streptococci, had the capacity to induce amyloid when combined with the paraffin oil and emulsifying agent. Group A streptococci, type 6, strain S43/101/7 were employed in combination with Arlacel A and Bayol F. Of 20 mice injected 3 developed amyloid in the spleen; the liver and kidney were not involved.

Time of appearance of amyloid. To determine approximately when the amyloid first could be detected 20 mice were injected weekly for from one to 10 weeks with the complete Freund adjuvant employing *M. butyricum*, and 2 mice were killed each week, 7 days after the last injection. Amyloid first appeared in the spleen following the 4th injection. All mice receiving more than 5 injections had amyloidosis. The amount of amyloid in the tissues increased with the number of injections. The renal lesion was not observed in this series until after the 10th injection. These results are in contrast with those of the caseinate method employed in other studies(8-12) for which at least 30 injections over a 6-week period are required for the earliest deposition of amyloid. Whether the number of injections or the time interval is most important for amyloid deposition is not answered by this experiment; but earlier workers(9,11,12) employing the caseinate method, observed that both the number of injections and duration of administration rather than the quantity of material introduced are the significant factors.

Summary. A method has been described for the induction of amyloidosis in mice by injection of the Freund-type adjuvant. The *M. butyricum*, or oil and emulsifying agent alone were not effective. *M. butyricum* with either the oil or emulsifying agent was capable of inducing amyloid. *N. asteroides* or Group A streptococci could be substituted for *M. butyricum* but the incidence of amyloidosis was less. The present method, as compared with previous ones, requires fewer injections, and it produces a higher incidence of amyloidosis in a shorter time.

1. Freund, J., *Am. J. Clin. Path.*, 1951, v21, 645.
2. Lipton, M. M., and Freund, J., *J. Immunol.*, 1953, v71, 98.
3. Thomson, K. J., Freund, J., Sommer, H. E., and Walter, A. W., *Am. J. Trop. Med.*, 1947, v27, 79.
4. Freund, J., Thomson, K. J., Hough, H. B., Sommer, H. E., and Pisani, T. M., *J. Immunol.*, 1948, v60, 383.
5. Gorer, P. A., *J. Path. and Bact.*, 1940, v50, 25.
6. Dunn, T. B., *J. Nat. Cancer Inst.*, 1944, v5, 17.
7. Freund, J., and Lipton, M. M., Proc. Soc. Exp. Biol. and Med., 1948, v68, 373.
8. Lucké, B., and Markley, L. A., *ibid.*, 1928, v25, 642.
9. Grayzel, H. G., Jacobi, M., Warshall, H. B., Bogin, M., Bolker, H., *Arch. Path.*, 1934, v17, 50.
10. Kuczynski, M. H., *Virchow's Arch. f. Path. Anat.*, 1922, v239, 225.
11. Jaffe, R. H., *Arch. Path.*, 1926, v1, 25.
12. Smetana, H., *Bull. Johns Hopkins Hosp.*, 1925, v37, 383.

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Heat Studies on a Thermophilic Bacteriophage.* (20809)

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Inactivation of bacteriophage by heat has been observed by several investigators, and seems to be a general phenomenon(1-3). Nanavutty(2), who exposed coli-dysentery phage to various temperatures and Smith and Krueger(3), employing vibrio-phage, found that their respective phages were not inactivated at a constant rate; heat inactivation curves (50° to 65°C) obtained by them were concave and became asymptotic with the time-axis. In contrast, the inactivation of a staphylococcus phage at various temperatures followed a monomolecular reaction and the temperature coefficient was characteristic of protein denaturation(4).

Koser(5,6) isolated a thermophilic phage from sewage-polluted river water and observed the optimum temperature for the lytic activity of the virus to be about 50°C, its ability to lyse the host at 57° to 58°C, its survival at 70°C for 30 minutes, and its destruction at 75°C. Heat resistance tests demonstrated that despite activity at higher temperatures, the phage was inactivated at about the same temperature (70° to 75°C) as the coli-dysentery phages. A thermophilic phage studied by Adant(7) was found to be active at 52°C, but was destroyed at 75°C when exposed for 30 minutes.

Recently we isolated from green house soil a bacteriophage capable of lysing a thermophilic bacterium at 65°C. Because the virus was active at high temperatures, heat inactivation studies were made since it was thought that it might be more resistant to heat than phages thus far reported. This investigation

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* Aided by a grant from the Division of Research Grants and Fellowships, U. S. Public Health Service.

was made to determine the rate at which the virus was destroyed and the temperature at which no further lytic activity was demonstrable.

Materials and methods. The host organism is a thermophilic bacterium designated No. 10 in a group of bacteria isolated from the hot springs of Yellowstone National Park and characterized by Marsh and Larsen(8). The optimum temperature for growth of this organism is 65°C, the same temperature at which the virus exhibits maximum activity.

The phage lysate was prepared in Trypticase broth (20 g Trypticase [Baltimore Biological Laboratory] per liter, at pH 7.0). The lysate was allowed to ripen for a few days in the refrigerator, then centrifuged at 4000 r.p.m. for 20 minutes to remove bacterial debris and finally passed through an ultrafine sintered glass filter. (When a Berkefeld filter was used, the titer of the filtrate was reduced 50%.)

Because of the high temperatures employed, a constant temperature oil bath was used in these studies. Small test tubes were prewarmed at the desired temperature and 0.5 ml aliquots of the lysate were introduced into these tubes which were immediately returned to the bath. At one-half hour intervals, samples were removed and diluted at once with Trypticase broth to stop the action of the heat. The residual active phage was determined by plaque count using the agar layer technic. Because of the higher temperature of incubation, 2.0% Trypticase agar was used in the plates and 0.9% to 1.0% Trypticase agar in the layer. An unheated aliquot was always plated simultaneously with the heated aliquots to determine the activity of the phage at the start of each run. Three runs were made at each temperature on consecutive days.

Results. The phage lysate was exposed to temperatures which ranged from 70°C to 100°C to determine the rate of inactivation as well as the temperature at which there was no further evidence of phage activity. When the logarithm of residual active phage was plotted against time of heating (Fig. 1), it was observed that straight lines were not obtained, indicating that inactivation did not follow a unimolecular reaction.

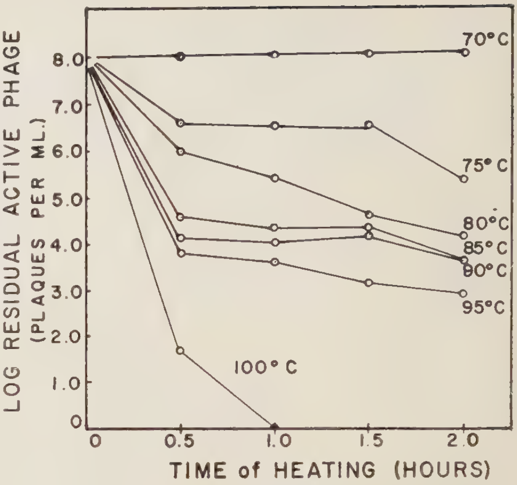

FIG. 1. Heat inactivation of a thermophilic bacteriophage in a broth lysate at pH 7.0.

At 70°C there was no appreciable decline in activity. After an exposure of two hours at 75°C, there remained about 340000 phage particles capable of producing plaques as compared to one hundred million per ml at 70°C. Two hours at 80°C reduced the number to 13000.

At 100°C there were but 50 plaques per ml detectable after the phage was exposed for thirty minutes with complete inactivation at the end of one hour. At temperatures of 85°C, 90°C and 95°C most rapid inactivation occurred during the first half hour, after which time the rate decreased quite markedly. Plaque-producing phage particles observed at 0.5 hour were 35000 at 85°C and 6000 at 95°C, while at the end of two hours there still remained 3500 and 1000 respectively per ml.

A precipitate was observed in the lysate within a few minutes after heating at 95°C. Its significance and character were not investigated. Even after the precipitate appeared, the phage was still active after a two hour exposure at 95°C. This would indicate that the substance precipitated exhibited little or no protective effect on the phage.

It was also noted that after exposure to the higher temperatures (85°C to 95°C) the plaques were considerably larger than at 65°C to 80°C, otherwise they appeared to be the same.

Discussion. It can be concluded from the data presented in the graph that the rates of inactivation of this thermophilic virus are very similar to those of the phage systems reported by Nanavutty(2) and Smith and Krueger(3). However, the temperatures at which the phage becomes susceptible to inactivation are much higher than those of the other reported phages. The virus is quite stable at 70°C but above this temperature the curves become concave. The most rapid inactivation occurs in the first half hour at temperatures above 80°C with a decided decrease in rate for the remainder of the period. There is no evidence of plaque formation after exposure to 100°C for 60 minutes and the virus is assumed to be completely destroyed.

This virus begins to be inactivated at about 75°C, at which temperature the thermophilic phages reported by Koser(5,6) and Adant(7) were completely destroyed. These data bear out the hypothesis that the virus used in this investigation is more heat resistant than any previously reported.

Summary. A thermophilic bacteriophage has been isolated which is inactivated by heat at a rate which is not constant but decreases with time. The phage remains viable after two hours exposure at 95°C, but is destroyed at 100°C, indicating that it is much more heat resistant than phages previously reported.

1. d'Herelle, F., *The Bacteriophage and Its Behavior,* The Williams and Wilkins Co., 1926, Baltimore, 292-296.

2. Nanavutty, S., *J. Path. and Bact.,* 1930, v33, 203.

3. Smith, L. S., and Krueger, A. P., Proc. Soc. Exp. Biol. and Med., 1952, v81, 371.

4. Krueger, A. P., *J. Gen. Physiol.,* 1932, v15, 363.

5. Koser, S. A., Proc. Soc. Exp. Biol. and Med., 1926, v40, 109.

6. Koser, S. A., *ibid.,* 1927, v41, 365.

7. Adant, M., *Compt. rend. Soc. Biol.,* 1928, v99, 1244.

8. Marsh, C. L., and Larsen, D. H., *J. Bact.,* 1953, v65, 193.

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Blood Eosinophil Levels in the Young and Adult Pyridoxine-Deficient Rat. (20810)

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The 24-hour rhythm of the blood eosinophils has been shown to synchronize with the 24-hour cyclic variations of the urinary excretion of ketosteroids(1,2) as well as with that of the blood lymphocytes(1,3). These correlations with currently accepted indices of adrenal function make possible the use of the blood eosinophil levels taken at a specific phase in their rhythm as an additional index of adrenal activity. A characteristic eosinophil level may be established for any species and strain provided constant conditions are maintained(1,4).

In the present experiment, the blood eosinophil levels of the pyridoxine-deficient male rat were determined throughout the progress of the deficiency. In order to evaluate the role of inanition in these changes, pair weighed and normal controls were also studied.

Materials and methods. Young animals. To develop an early and severe pyridoxine deficiency(5), on the 15th day of age litters were divided into 3 groups. One-third of the males were placed with lactating mothers receiving a pyridoxine-deficient ration (Table I) and the remaining males were left with mothers receiving the same diet containing pyridoxine. On the 21st day of age the young animals were placed in individual metabolic cages. Three groups of 8 animals per group were fed as pyridoxine-deficient, pair weighed and *ad libitum.* The young animals received a high protein diet (Table I) which resulted in a more severe pyridoxine-deficiency than lower protein levels would have induced. A complete vitamin mixture (Table I) was fed 3

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TABLE I. Purified Diets Fed to Lactating Mothers, Young, and Adult Rats.

Constituents	Lactating mothers	Young rats‡	Adult rats‡
		%	
Casein	36	50	24
Hydrogenated vegetable oil	4	5	10
Salt mix(5)	4	4	4
Sucrose	54	41	62
Vit. B mixture*	1	0	0
Vit. A & D oil†	1	0	0

* Each kg of diet fed to mothers supplied the following vitamins in mg amounts: thiamine hydrochloride, riboflavin, pyridoxine, and folic acid, 2.0 each, calcium pantothenate and para-aminobenzoic acid, 10 each, niacin amide, 6.7, inositol, 250, biotin 0.27, and choline, 500.

† Each kg of diet contained the fat soluble vitamins in the following amounts: vit. A, 10000 I.U., vit. D, 1000 I.U., and mixed tocopherols, 100 mg, dissolved in 9 g of cottonseed oil.

‡ Water soluble vitamins dissolved in 20% aqueous ethanol and supplied each rat daily the following amounts in mg: thiamine hydrochloride, riboflavin, pyridoxine and folic acid, .02 each, calcium pantothenate and para-aminobenzoic acid, .10 each, niacin amide, .07, inositol, 2.5, biotin, .002, and choline, 5.0. Vit. A and D and mixed tocopherols were dissolved in vegetable oil and were supplied separately in amounts of 1000 I.U., 100 I.U. and 3 mg/rat/wk.

times weekly in individual cups; the deficient group received no pyridoxine in this mixture.

Adult animals. Adult male stock rats at an average age of 103 days were divided into the 3 groups mentioned above containing 8 animals each. They were given a diet containing 24% casein (Table I). The deficiency condition was induced by the addition of 30 mg of desoxypyridoxine per kg diet mixed directly into the ration of the deficient group and by the omission of pyridoxine from their vitamin mixture. *Eosinophil counting technic.* In young rats eosinophil counts were determined at weekly intervals for 9 weeks. In adult rats the circulating eosinophil levels were observed at irregular intervals from the 25th to 46th day. The eosinophil counts of 8 animals per group were used in the young and adult series. Blood was drawn from the tail vein into a white blood pipette to the 0.5 mark and diluted with phloxine eosinophil diluent (6). If a variation of greater than 10% occurred between counts of duplicate pipettes, the values were discarded. For young rats, both chambers of a Levy hemocytometer, and for adult rats, one chamber of a Fuchs-Rosenthal hemocytometer were counted for each of the 2 pipettes per animal. In order to eliminate diurnal variations all counts were completed during the hours of 9:00 a.m. and 12:00 noon in the same room in which the animals were housed in order to maintain the environmental conditions as constant as possible(4).

Results. Young animals. The average body weight gain at the termination of 9 weeks on the regimen was 68% for the deficient and pair weighed groups, whereas, the *ad libitum* group gained 450% above their respective weights on the 21st day of age. In the deficient animals acrodynia appeared in the 3rd and 4th weeks. Xanthurenic acid was detectable in the urine from the 14th to 16th day on the regimen. The rapidly growing *ad libitum* animals (Fig. 1) showed a continuous steep ascent of the circulating eosinophils from the 1st week with 27 ± 5 per mm^3 to 268 ± 38 per mm^3 in the 8th week when no further rise in the level occurred. In contrast, the deficient and pair weighed groups had a comparable but gradual rise in the eosinophil levels throughout the course of the experiment. The last two mentioned groups showed lower eosinophil levels ($p = \text{or} < .01$) than did the *ad libitum* group. First evidence of these depressed levels was observed in the

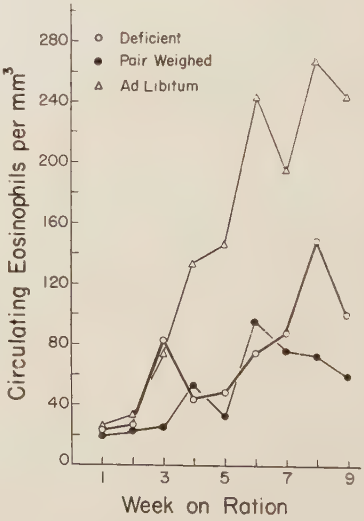

FIG. 1.
Circulating eosinophil levels in growing rats.

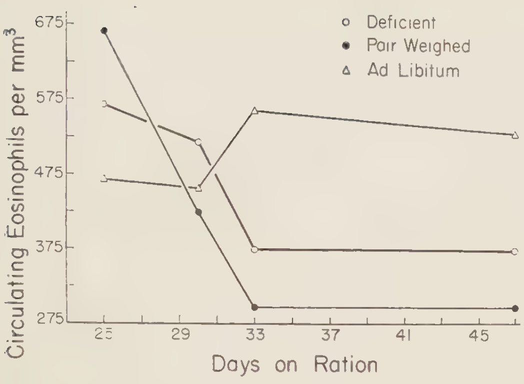

FIG. 2. Circulating eosinophil levels in adult rats.

deficient group at the start of the 4th week and in the pair weighed group at the beginning of the 3rd week. These significantly lower circulating eosinophil levels continued for the duration of the experiment.

Adult animals. The adult deficient and pair weighed groups lost 29% while the *ad libitum* group gained 3.5% of their initial body weight during the 8 weeks on the dietary regimen. Signs of acrodynia appeared within 2 weeks and progressively became more severe. On the 25th day of the experiment (Fig. 2) the eosinophil levels of the 3 groups were equal ($p = .3$ to .7). The *ad libitum* group showed no significant rise differences in the blood eosinophil levels throughout the period of observation. On the other hand, the eosinophil levels in the deficient and pair weighed groups were characterized by an equal and gradual fall until significantly lower levels ($p =$ or $< .01$) occurred simultaneously on the 33rd day and continued to remain low through the 46th day.

Discussion. These findings show that during rapid growth the eosinophil level in young normal animals ascends steeply until the 63rd day of age. In contrast, normal adult animals whose growth was insignificant maintained a constant eosinophil level for the duration of the experimental period. Clearly then, growth alone in the normal young rat caused an increase in the number of blood eosinophils.

An equal degree of endogenous eosinopenia gradually occurred in the young and adult pyridoxine-deficient animals as compared with their respective pair weighed controls. Inanition in the young calorie-restricted animals, whose growth was markedly retarded, produced a greater fall in the circulating eosinophils than it did in the adult animals similarly restricted. From these results it is inferred that inanition, not pyridoxine deficiency *per se,* was the specific cause of the eosinophilic leucopenia. Studies of chronic inanition have usually demonstrated a normal or hyperfunctional adrenal(7-9) although adrenal hypofunction has been reported by one group of workers(10). During calorie restriction in rodents, a greater amplitude without a shift of the 24-hour eosinophil rhythm(7), a greater deposition of liver glycogen under the influence of hypoxia(8), and a gradual development of a lymphopenia(9) are supporting evidences of a functional as well as a hyperfunctional adrenal. We also have demonstrated previously(11) that pair weighed and pyridoxine-deficient rats were capable of eosinopenic responses to stimuli equal to those of their *ad libitum* controls.

The depression of the circulating eosinophils in the pair weighed and deficient young and adult animals in the experiment here reported probably indicated an equal and augmented secretion of the adrenal 11-oxysteroid hormones comparable to that seen in cases of an endogenous eosinopenia(12). Since experiments were not performed on adrenalectomized animals, it is impossible to state categorically that the depression of the eosinophils was mediated solely by the adrenals.

Summary. The blood eosinophil levels of pyridoxine-deficient and pair weighed young and adult male rats were of the same order of magnitude and were significantly lower throughout the experiment than those of full-fed normal controls. These findings indicated that inanition alone was the cause of the chronic endogenous eosinopenia, therefore, excluding lack of pyridoxine *per se* as the agent.

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1. Halberg, F., *Lancet,* 1953, v73, 20.

2. Pincus, G., Romanoff, L. P., and Carlo, J., *J. Clin. Endocrinology,* 1943, v3, 195.

3. Elmadjian, F., and Pincus, G., *J. Clin. Endocrinol.,* 1946, v6, 287.

4. Halberg, F., Bittner, J. J., and Visscher, M. B., *Blood,* 1946, v6, 832.

5. Hubbell, R. B., Mendel, L. B., and Wakeman,

A. J., *J. Nutrition,* 1937, v14, 273.

6. Speirs, R. S., and Meyer, R. K., *Endocrinology,* 1949, v45, 403.

7. Halberg, F., and Visscher, M. B., *ibid.,* 1952, v51, 320.

8. Hurley, L. S., and Morgan, A. F., *J. Biol. Chem.,* 1952, v195, 583.

9. Shukers, C. F., and Day, P. L., *J. Nutrition,* 1943, v25, 511.

10. Mulinos, M. G., and Pomerantz, L., *Endocrinology,* 1942, v31, 276.

11. Butler, L. C., and Morgan, A. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 655.

12. Thorn, G. W., Forsham, P. H., Recant, L., and Hills, G. A., *J. Clin. Endocrinol.,* 1948, v8, 589.

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Action of Fibrinolysin (Plasmin) on Proteins.* (20811)

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Previous workers(1,2) have found that the products of the action of fibrinolysin (plasmin) on fibrinogen and fibrin are of high molecular weight. In the method of Ungar and coworkers(3) for the assay of fibrinolysin, unchanged fibrinogen is precipitated as fibrin and the material remaining in the filtrate is measured. If the fibrinogen is precipitated with trichloroacetic acid (TCA), the reaction products are precipitated along with the unchanged fibrinogen and cannot be found in the filtrate. Similarly, the soluble material formed by the action of fibrinolysin on fibrin is largely precipitable by TCA. The action of rennin upon casein is similar in some respects to that of thrombin upon fibrinogen. After exposure to the action of fibrinolysin if unchanged casein is precipitated with rennin, there is more soluble material present than when the casein is precipitated with TCA. These observations confirm the conclusions of previous workers(1,2) that the product of the action of fibrinolysin consists of proteins or peptides of high molecular weight.

Methods. The buffer in all cases was that of Ungar and coworkers(3) consisting of 0.15 M phosphate (sodium salts) of pH 7.4 in 0.1 M NaCl. Solutions of 0.1% bovine fibrinolysin[†] and 0.2% bovine fibrinogen (Armour) in buffer were made up daily as needed. Casein (Pfanstiehl) was dissolved in sufficient buffer to give a 2% solution by heating 10 minutes at the temperature of boiling water, cooled, made up to the original volume with distilled water, and filtered. It was stored in the refrigerator as long as one week. Thrombin[†] was used as a solution in isotonic saline containing 300 units per ml. It was stored in the frozen state for periods up to 2 weeks. Rennin (Nutritional Biochemicals) was dissolved in distilled water as needed to give a 0.1% solution. Fibrinolysin was assayed for its activity for fibrinogen and fibrin by the method of Ungar *et al.*(3). Activity for casein was determined similarly using rennin as the coagulant. The substrate (1.0 ml of 2% solution) was acted on by various concentrations of fibrinolysin in a total volume of 4.5 ml of buffer. After one hour at 37°C sufficient 0.1 M citric acid (1.1 ml) to bring the pH to about 5.3 and 0.1 ml of rennin solution were added. After 10 minutes at 37°C, the pH was adjusted to about 4.8 (0.3 ml of citric acid). The pH's were chosen to approximate the optima found by Lundsteen(4) for enzymatic action of rennin on casein and for coagulation of the product. After 30 minutes at 37°C the precipitates were filtered, and the optical density of the filtrates was read at 280 mμ against blanks without casein. Readings were corrected for the reading on casein with fibrinolysin absent. Although the isoelectric point of casein was approached at pH 4.8 and its solutions became milky in

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[†] We are indebted to Dr. E. C. Loomis of Parke, Davis and Co. for this preparation.

TABLE I.

	Optical density change in filtrate/mg fibrinolysin/hr		
Substrate	Thrombin or rennin coagulation method	TCA precipitation method	Ratio
Fibrinogen	1.71	.013	131.5
Fibrin	.106	.022	4.8
Casein	.248	.076	3.3

appearance, no coagulation occurred when rennin was absent.

To determine activities using TCA as precipitant, substrate was incubated with enzyme for one hour at 37°C under the above conditions. Sufficient TCA was then added to give a final concentration of 5%. After 30 minutes the precipitates were filtered off and the filtrates read at 280 mμ against blanks without enzyme. Experiments on each substrate were run simultaneously using aliquots from the same solutions of enzyme and substrate and varying only the method of precipitation of protein. Activity appeared to be linear with enzyme concentration over the range investigated in each case. Differences in range of enzyme concentration and time of incubation depending on the substrate and the method of precipitation were necessitated by the limitations of the spectrophotometer. Fibrinogen was incubated with 0-0.4 mg of fibrinolysin for 20 minutes when it was precipitated with thrombin, whereas it was incubated one hour with 0-3.0 mg of enzyme when it was precipitated by TCA. The enzyme was assayed for activity on fibrin at levels of 0-2.0 mg, the incubation time being one hour. When unchanged casein was precipitated with rennin fibrinolysin was assayed at levels of 0-1.0 mg, compared to a range of 0-3.0 mg when TCA was used. When TCA was the protein precipitant the final volumes were 6.3 ml for fibrinogen and 6.6 ml for fibrin, rather than 6.0 ml, as in all other cases. Optical density differences were corrected to allow for the additional dilution.

Results. Table I gives the average change in optical density of filtrate per hour per milligram of fibrinolysin. It can be seen that the products of the action of fibrinolysin on fibrinogen, which are non-coagulable by thrombin, are almost entirely precipitated in 5% TCA. They must therefore consist of peptides of high molecular weight. They could not be differentiated from fibrinogen by paper chromatography or paper ionophoresis under several conditions, and preliminary light scattering measurements indicate that their average molecular weight is of a comparable order of magnitude to that of fibrinogen. As the fibrinolysin preparation is not homogeneous, the very small amount of product which is soluble in TCA may result from proteolytic activity of a contaminating enzyme upon fibrinogen or the initial reaction products. Several authors(5-8) have found evidence for a multiplicity of enzymes with peptidase, proteolytic, or fibrinolytic activity in various blood sera.

The activity of the enzyme for casein and for fibrin is of a different magnitude than its activity for fibrinogen. Ungar and coworkers (9) have already demonstrated this difference in activity for fibrinogen and fibrin. This may be because of a difference in affinity of the enzyme for these substrates or because they are acted on by an enzyme present as a contaminant in low concentration. The insolubility of fibrin may also account for the relatively poor activity of the enzyme. The products from fibrin, as determined by lysis, and from casein, as determined by noncoagulability with rennin, are largely precipitated by TCA, as shown in Table I. However, about 20% of each product is soluble in 5% TCA, if the reaction rate can be assumed constant and linear with enzyme concentration. The portion of lower molecular weight may result either from simultaneous splitting of smaller fragments from the original protein by the principal enzyme or an impurity, or by further splitting of the primary products. Several proteolytic enzymes have been shown(10-13) to form primary products of high molecular weight, which are then broken down to smaller fragments. The action of fibrinolysin may well be similar in nature, except that the products of high molecular weight from fibrinogen do not readily undergo further reaction.

Summary. By the action of fibrinolysin fibrinogen is converted to products which are not coagulated by thrombin, but which are precipitated by TCA. The enzyme is much less active for fibrin and casein. The former is lysed to give a product only about 20% of which is soluble in TCA. The product from casein, which is not coagulated by rennin, is also largely precipitated by TCA, although soluble to the extent of about 20%.

1. Holmberg, C. G., *Ark. Kemi, Mineral Geol.*, 1944, v17A, No. 28.

2. Seegers, W. H., Nieft, M. L., and Vandenvelt, J. M., *Arch. Biochem.*, 1945, v7, 15.

3. Ungar, G., Damgaard, E., and Hummel, F. P., *Endocrinology*, 1951, v49, 805.

4. Lundsteen, E., *Biochem. Z.*, 1938, v295, 191.

5. Holman, H. R., White, A., and Fruton, J. S., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 196.

6. Mole, R. H., *J. Path. Bact.*, 1948, v60, 413.

7. Cliffton, E. E., and Cannamela, D. A., *Blood*, 1953, v8, 554.

8. ———, *J. Applied Physiol.*, 1953, v6, 42.

9. Ungar, G., Damgaard, E., and Hummel, F. P., *J. Exp. Med.*, in press.

10. Petermann, M. L., *J. Biol. Chem.*, 1942, v144, 607.

11. ———, *J. Phys. Chem.*, 1942, v46, 183.

12. Moring-Claesson, I., *Biochim. et Biophys. Acta*, 1948, v2, 389.

13. Desnuelle, P., Rovery, M., and Bonjour, G., *Biochim. et Biophys. Acta*, 1950, v5, 116.

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The Depolarization of Nerve by Organic Compounds.* (20812)

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One of the perplexing aspects of the action of narcotic agents on nerve has been noted by Laget *et al.*(1), by Bishop(2), and by Brink(3). This is the fact that while the lower alcohols of the paraffin series depolarize nerve before a conduction block ensues, the higher alcohols and many other narcotic agents block conduction before there is any detectable depolarization. Indeed, there seems to be a slight increase in the membrane polarization associated with the action of the higher alcohols and with cocaine. In order to investigate this problem in more detail, we have measured the depolarization produced by a variety of organic reagents, and we have attempted to explain the results obtained in terms of a model.

Methods. In most experiments, bullfrog (*R. catesbiana*) sciatic nerves were used, although a few measurements were made on *R. pipiens* nerves. The general arrangement of the chamber for mounting nerves was similar to that used by Lorente(4). In most cases, the depolarization produced by 0.11 M KCl at one region along the nerve was compared with the depolarization produced by an experimental solution at another region along the nerve. Such measurements permitted the calculation of a depolarization rate relative to the rate of KCl depolarization as a standard. Measurements were made with a recording potentiometer so arranged as to record the potential between the KCl-Ringer segment and between the narcotic agent-Ringer segment at 16 sec. intervals. The sensitivity of this instrument with the nerve in the external circuit was about 0.1 mV. and the current drawn at balance, 10^{-9} amperes. The usual technic for measurement was to mount a nerve, previously dissected and kept in Ringer solution for 2 hours, in the chamber with Ringer in all electrodes. This was a check for any initial depolarization. Next, KCl, 0.11 M was added to electrode 1. Ringer to electrode 2, and test agent dissolved in Ringer to electrode 3. Recording was initiated, and continued usually for several hours after which all electrodes were changed to Ringer, and recording was continued to follow the course of repolarization. Throughout the course of the experiments, 95% O_2-

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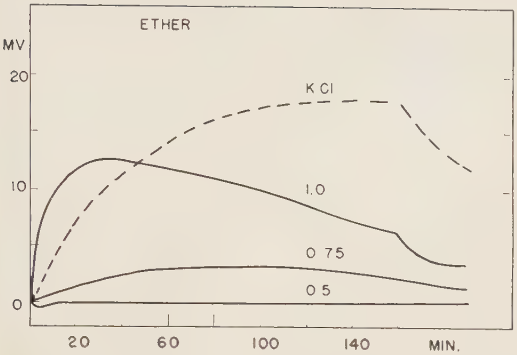

FIG. 1. Depolarization produced by ethyl ether at various thermodynamic activities plotted against time. Dotted line gives time course of KCl depolarization, measured on another segment of same nerve. At 160 min. solutions were changed to Ringer, and repolarization commenced.

5% CO_2 was allowed to flow slowly through the chamber. The ambient temperature was 20 ± 1°C, and the composition of the Ringer solution: NaCl 116 mM, KCl 2 mM, $CaCl_2$ 1.8 mM.

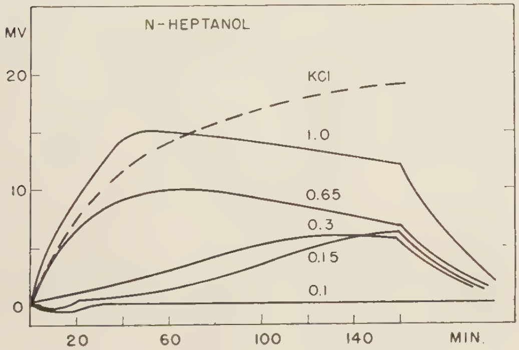

FIG. 2. Depolarization produced by n-heptanol at various thermodynamic activities plotted against time. Dotted line gives time course of KCl depolarization measured on another segment of same nerve. At 160 min. solutions were changed to Ringer, and repolarization commenced.

Results. A series of experiments was undertaken to determine the time course of depolarization produced by various organic substances, *viz.* diethyl ether, dichloromethane, chloroform, acetone, dioxane, n-pentane, and n-heptanol, as compared with KCl. Fig. 1 and 2 show some records for the depolarization produced by ether and by n-heptanol respectively at various concentrations. Table I gives a summary of the values obtained for other substances. The thermo-dynamic activity coefficients (γ_{H_2O}), for sparingly water soluble substances were computed by assuming a linear relationship between activity and saturation of an aqueous solution. Because the activities employed were generally high (greater than 0.5), this approximation is felt to involve no great error. For acetone, the activity at the various concentrations used was computed from vapor pressure-composition data as given in International Critical Tables. For dioxane, activity figures were approximated from freezing point depression data (Mellan(5)).

It appears clear that with the exception of n-pentane, which does not depolarize nerve, of chloroform which only rarely can produce a slight depolarization and n-heptanol which depolarizes at rather low values for the thermodynamic activity, all other substances tested depolarize nerve appreciably only at concentrations representing a thermodynamic activity greater than 0.5. This figure is to be compared with the thermodynamic activity for nerve block, $A_{nar} = 0.05$. Several features of the time course of depolarization are worth comment. The rate of depolarization by a saturated aqueous solution of ether is about 5 times faster than that produced by isosmotic KCl. Furthermore, the depolarization produced by ether is not maintained, but declines gradually to about half its maximum

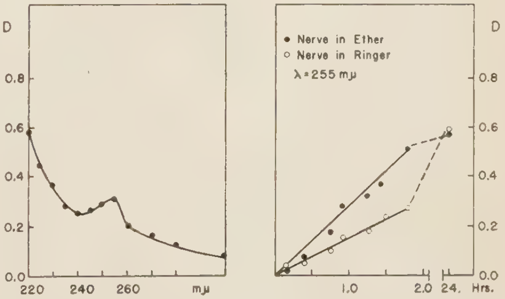

FIG. 3. On left is plotted optical density vs. wavelength for difference between a sol. containing 1.0 M ether in Ringer, plus a frog sciatic nerve, as compared with a Ringer sol. containing a frog nerve. Curves were obtained after one hour's immersion of nerve. Volume in both cases was 5 ml, and ether added to control Ringer sol. before measurement. On right, optical density plotted at 255 mμ vs. time a 1 M ether sol. was in contact with nerve; curve is relative to a control nerve in Ringer.

TABLE I. Depolarization of Frog Nerve by Various Substances.

	Concentration		Initial rate of depolarization, mV/min.		Ratio:
Substance	M/L	A	Narcotic	KCl	Narcotic/KCl
Ethyl ether	1.0	1.0	2.5	.5	5
	1.0	1.0	2.0	.4	5
	1.0	1.0	2.0	.4	5
	1.0	1.0	2.3	.5	5
	.8	.8	.7	.4	2
	.75	.75	.0	.4	—
	.75	.75	.02	.4	—
	.75	.75	.07	.4	—
Dichloromethane	.24	1.0	.8	.4	2
	.24	1.0	.8	.4	2
	.24	1.0	.7	.4	2
	.19	.8	.0	.5	—
1,4 Dioxane	2.5	.3	.02	.6	—
	2.5	.3	.05	.5	.1
	3.0	.4	.13	.4	.3
	1.3	.2	.0	.5	—
Acetone	5.2	.4	.9	.4	2
	3.0	.3	.5	.4	1
	1.6	.18	.0	.5	—
n-Pentane	.005	1.0	.0	.4	—
	.005	1.0	.0	.4	—
Chloroform	.084	1.0	.0	.5	—
n-Heptanol	.0078	1.0	1.0	.4	2.5
		1.0	1.0	.7	1.4
		1.0	1.0	.4	2.5
		1.0	1.1	.4	2.6
		1.0	.9	.7	1.3
		.65	.8	.5	1.6
		.3	.6	.4	1.5
		.15	.6	.4	1.5
		.10	.0	.5	—
		.05	.0	.4	—

value. Recovery from such depolarization is seldom complete, and after 2 hours there is a permanent depolarization about 5 mV below the initial level of the membrane potential. There is a rough equivalence between the initial rate of depolarization and the maximum value of the demarcation potential. The former measurement has been taken by us as being a more reproducible index of the action of substances in producing depolarization.

We have made some measurements in which a nerve was immersed in ether-saturated Ringer solution for some time, and a control paired nerve was kept in Ringer alone for the same period. The solutions were then placed in quartz cuvettes, and the ultraviolet absorption curve of each determined, using ether-saturated Ringer as a blank in one case, and Ringer in the other. The results of such experiments are shown in Fig. 3 and in Table II. In all cases where there was depolarization produced by an organic substance, there was in increased release of substances absorbing light at 255 mμ. An observation such as this can be related to the report that frog muscle loses a great deal of nitrogenous material when it is first immersed in Ringer solution(6), and that this loss is decreased by raising the external K^+ concentration and increased by anoxia. Since nerves depolarized in KCl do not show this increased release of material absorbing at 255 mμ, one may conclude that there is more than just a quantitative difference between the depolarization produced by narcotic agents and by KCl.

Discussion. The interpretation of the experimental results obtained presents two different problems. In the first, some explana-

TABLE II. Effect of Organic Substances on Loss of Material from Nerve.

Substance	Time of treatment of nerve, hr	Conc., M/L	% transmission at 255 mμ. Narcotic minus Ringer nerve sol.
KCl	18.5	.11	.5
Pentane	18.0	.005	1.3
Dioxane	18.0	3.0	28.2
Ether	1.0	1.0	50.0
Acetone	17.0	5.2	35.8
Acetone	17.0	3.0	32.5

tion must be found for the fact that ether at 0.75 M gives either a slight depolarization or none at all while at 1.0 M, it gives a depolarization many times faster than that of KCl. Such an observation is in marked contrast with studies on the kinetics of chemical reactions where inhibition or acceleration of the rate by various reagents takes place over a range of concentration of 2 log units. The second problem is to explain the markedly different concentrations (or activities) necessary for depolarization by the alcohols, as contrasted with less polar organic molecules. An explanation of the first point, namely the narrow range of concentration of aqueous solutions of ether that produce depolarization must lie in some physical change in such a solution as the concentration of ether is changed from 0.8 to 1.0 M. Such a change, taking place mainly in this concentration range, is the variation of the surface tension of the solution. Ether is not a particularly surface active substance, and low concentrations of ether in water have practically no effect on the surface tension of the solution. Dichloromethane has less, and chloroform even less surface activity; indeed a saturated aqueous solution of chloroform has a surface tension only 13 dynes/cm less than that of pure water. Pentane is completely without surface activity, while both dioxane and acetone are appreciably surface active only at very high aqueous concentrations. Fig. 4 summarizes our measurements and correlates them with the surface tension depression produced by the various organic reagents. If the depolarization produced by these substances were somehow related to the amount of each substance partitioned to the cell membrane, we should expect(3) that they would all act at some constant value of thermodynamic activity. Actually, at an activity of 1.0, ether depolarizes rapidly and chloroform not at all. The alcohols, by contrast with these other agents, are surface active even at low concentrations and the interpretation of the depolarization produced by the alcohols will be more complex.

The application of the notion of thermodynamic activities (A_{nar}) in narcosis(7,8) has been very helpful in explaining the aqueous concentrations required for nerve block(3); the determination of the activities for the alcohols in aqueous solutions is based on the data of Butler(9,10) who, however, has shown that the first 3 n-alcohols exhibit greater deviations from ideality when dissolved in benzene, than when dissolved in water. This is also true in heptane(11). Since a constant thermodynamic activity for nerve block implies an ideality of behavior of the narcotic agent in the non-aqueous system to which it is supposed to be partitioned, it is apparent that one can expect anomalous behavior from the alcohols, in that higher aqueous activities will be required to produce a given mole fraction in the non-aqueous phase. Heptanol, which we have studied, behaves ideally enough

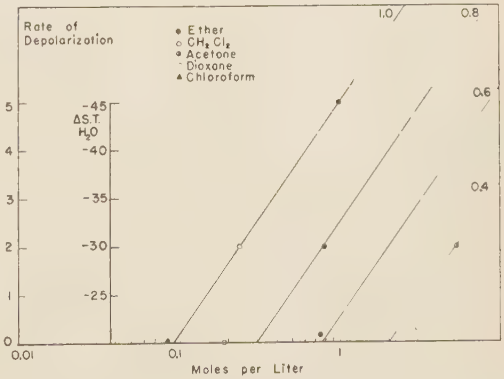

FIG. 4. Rate of depolarization, relative to KCl, plotted against aqueous concentration in moles/liter of various organic substances. Lines represent various thermodynamic activities for a particular solute, and are intended to show that a certain concentration is required to produce depolarization at any given activity. Superimposed ordinate $\Delta S.T._{H_2O}$ is the depression of surface tension of water in dynes/cm that a solution of given substance causes, at concentration shown on abscissa.

so that conduction is blocked at $A_{nar} = 0.07$ while depolarization starts at $A_{dep} = 0.2$-.3. The lower alcohols behave less ideally in benzene, and more ideally in water. For n-propanol, the activity coefficients in both benzene and water are the same, 15, while at the interface benzene:water it is probable that the C_3H_7-part of propanol has unit activity coefficient, as does the –OH part of the molecule in water. Such a notion finds confirmation in a study(12) showing that alcohols from C_6-C_{12} at equal concentrations show equal interfacial tension reductions at the benzene:water interface. The Traube rule does not hold for this interface, suggesting, indeed, that there is a constancy of activity coefficient from one alcohol to another. We might, therefore, expect that the alcohols will show equal degrees of depolarization at equal aqueous concentrations, while narcosis will demand equal thermodynamic activity of the narcotizing molecules (as a first approximation). Just how these various factors are related, is illustrated by Fig. 5, where we have combined the data of Laget(1) on depolarization and narcosis of frog sciatic nerve, with our own for the depolarization produced by heptanol. The line X_{dep} represents the constant aqueous mole fraction necessary for a depolarization of about 2 mV, the line $X_{nar}^{H_2O}$ represents the aqueous mole fraction of the alcohols necessary for narcosis (conduction block). The curve $X_{nar}^{C_6H_6}$ is the computed mole fraction of alcohol in benzene for each aqueous mole fraction. The line X_{nar} represents the assumed threshold mole fraction required for narcosis and is drawn constant to butanol, and then rises. Methanol and ethanol have non-aqueous mole fractions below those necessary for narcosis but both alcohols have aqueous mole fractions well above the non-aqueous.

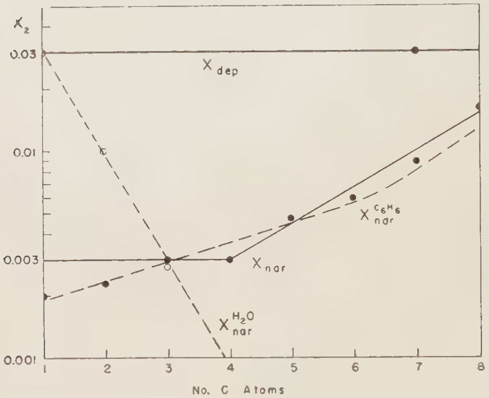

FIG. 5. Dotted line $X_{nar}^{H_2O}$ represents aqueous concentrations (as mole fraction) necessary for narcosis of frog sciatic nerve with various n-alcohols shown as abscissae. Dotted line $X_{nar}^{C_6H_6}$ represents computed mole fractions in benzene that give narcosis. Data are from Laget *et al.*(loc. cit.). Solid line X_{nar} represents assumed mole fraction required for narcosis. Initial value is 0.003 and is constant through n-butanol. This places the points for methanol and ethanol below the necessary mole fraction for narcosis. Line X_{dep} represents mole fraction necessary for depolarization of a certain degree. The point for methanol (open circles indicate aqueous mole fractions, solid circles benzene mole fractions) and the point for heptanol are experimentally determined. We are unable to compare the methanol with heptanol depolarizations directly, but curve is drawn parallel to abscissa on the assumption that tendency to accumulate at aqueous:non-aqueous interface is a constant for all alcohols. Other X_{dep} curves could be drawn, parallel and below the 0.03 mole fraction curve, these would represent lesser degrees of depolarization.

The data of Wilbrandt(14) on the effects of the dialkylamines on nerve depolarization suggest that surface activity of a compound is a factor of importance since dimethyl amine is without surface activity and does not depolarize while diamyl amine is both surface active and is equivalent to K^+ in depolarizing power. Agents which are highly surface active such as the salts of fatty acids also depolarize (13) and unmyelinated fibers appear to be more easily affected than do myelinated fibers. The data in the literature, and our present observations are not inconsistent with the view that the depolarization of nerve fibers can be produced in three, experimentally distinct, ways: (a) by anoxia, or metabolic poisons which, by interfering with sodium pumping, produce a slow depolarization; (b) KCl, by abolishing the K^+ gradient across the membrane; (c) by surface active agents which act, presumably, by disorganizing the membrane.

Summary. Depolarization produced by a variety of organic reagents acting on frog sciatic nerve has been measured. With the alcohols, the fact that the lower alcohols depolarize before blocking nerve, whereas higher

alcohols block conduction before depolarizing, is referable to the abnormally high activity coefficients of methanol and ethanol in non-aqueous solvents, and hence to the high aqueous mole fractions necessary for narcosis. While the alcohols depolarize at aqueous activities of about 0.15-0.30, other organic substances, of a relatively non-polar nature, depolarize only at activities of about 0.8. Such substances show a surface tension depression similar to that of the alcohols at these elevated thermodynamic activities. The distinction is made between narcosis, which requires a given mole fraction of a substance in the non-aqueous phase of the fiber, and depolarization which requires a given depression of the interfacial tension.

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1. Laget, P., Posternak, J. M., and Mangold, R., *Méchanisme de la Narcose, CNRS,* Paris, 1951.

2. Bishop, G. H., *J. Cell. Comp. Physiol.,* 1932, v1, 177.

3. Brink, F., *Nerve Impulse,* Macy Foundation, New York, 1951.

4. Lorente de Nó, R., *Studies from the Rockefeller Institute for Medical Research* 131, 1947.

5. Mellan, I., *Industrial Solvents,* Reinhold, New York, 1950.

6. Fenn, W. O., *J. Cell. Comp. Physiol.,* 1935, v6, 469.

7. Ferguson, J., *Proc. Roy. Soc.,* 1939, vB127, 387.

8. ———, *Méchanisme de la Narcose, CNRS,* Paris, 1951.

9. Butler, J. A. V., and Harrower, P., *Trans. Faraday Soc.,* 1937, v33, 171.

10. Butler, J. A. V., *ibid.,* 1937, v33, 229.

11. Smyth, C. P., and Engel, E. W., *J. Am. Chem. Soc.,* 1929, v51, 2646.

12. Hutchinson, E., *J. Coll. Sci.,* 1948, v3, 219.

13. Höber, R., *Compt. Rend. Lab. Carlsberg,* 1937, v22, 205.

14. Wilbrandt, W., *J. Gen. Physiol.,* 1937, v20, 519.

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Influence of Protein Intake on Magnesium Requirement during Protein Synthesis.* (20813)

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The deleterious effect of magnesium deficiency on protein synthesis was reported by Menaker and Kleiner(1). At a 9% level of protein intake (150 to 160 mg N daily), their rats gained about 40% more in 10 days on a complete ration than on a magnesium-deficient one (30.5 vs 21 g) with virtually isocaloric intake. They concluded that magnesium deficiency is approximately as deleterious to protein synthesis as is potassium deficiency, since Cannon *et al.*(2) had found that rats, on complete rations of 216 mg N daily, gain about 35% more in 10 days (about 46 g vs 34 g) than on an essentially isocaloric potassium-deficient ration. Frost and Sandy (3) recently reported that on 120 mg N daily, rats gain virtually as much on a Mg-deficient as on a complete ration the first few weeks, but in 11 weeks rats on a complete ration gain 25 to 30% more weight (about 90 g vs 70 g) than those on a Mg-deficient one. At this low level of N-intake, their rats on a complete ration gain less in 10 days than Menaker's and Kleiner's rats on a Mg-deficient ration of 150-160 mg N daily. An analysis of these data led the author to test whether the Mg requirement is influenced by the level of protein intake.

Method. 24 female Wistar albino rats, depleted 20-25% below initial 250 g (approx.), were divided into 4 groups. Group 1 received 7 g casein per 100 g basal ration plus complete mineral supplement. Group 2 received same basal ration (7% protein) but Mg-deficient supplement. Group 3 received 14 g casein per 100 g basal ration (14% protein) plus complete mineral supplement. Group 4 received this basal ration (14% protein) but

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* This work was aided by funds from the American Cancer Society.

TABLE I.
Effect of Magnesium Deficiency on Weight Gain at Different Levels of Protein Intake.

Protein level (%)	Mineral supplement	No. rats	Avg total food consumed (g)	Avg total wt gain*	Gain (g) / Food (g)	Food efficiency ratio†
7	Complete	6	120	14.3 ± 1.7	.119	.95
7	Deficient	6	109	12.3 ± 1.3	.113	
9‡	Complete	4	112	30.5 ± 2.3	.272	.71
9‡	Deficient	4	108	21 ± 1.5	.194	
14	Complete	6	117	36 ± 2.0	.308	.23
14	Deficient	6	80	5.8 ± .7	.072	

* Mean ± stand. error of mean. † Avg total wt gain (deficient)/avg total wt gain (complete). ‡ Menaker and Kleiner(1).

Mg-deficient supplement. Basal ration contained, per 100 g, 7 g (Groups 1 and 2) or 14 g (Groups 3 and 4) purified casein(1), 78 g (Groups 1 and 2) or 71 g (Groups 3 and 4) cane sugar, 7 g corn oil, 2 g U.S.P. cod liver oil, 5 g non-nutritive cellulose fiber, and 1 g mixture of B-complex vitamins in sucrose (2). Four g of complete mineral mixture (Hawk-Oser, Osborne-Mendel) were added to each 100 g basal ration fed Groups 1 and 3. Groups 2 and 4 received an amount of mineral supplement equivalent to that of Groups 1 and 3 but lacking Mg; K_2SO_4 and Na_2CO_3 were substituted in the supplement fed Groups 2 and 4 for the corresponding Mg salts in the supplement fed Groups 1 and 3. These diets were fed 10 days.

Results. Group 3 (14% protein, complete mineral) gained far more in 10 days than Group 1 (7% protein, complete mineral), but Group 4 (14% protein, Mg-deficient) gained considerably less than Group 2 (7% protein, Mg-deficient). Thus, with complete mineral supplement, the rate of protein synthesis (as measured by weight gain) was over twice as great at the higher protein intake as at the lower protein intake. But when Mg was omitted at both levels of protein intake, the rate of protein synthesis was over twice as great at the *lower* level of protein intake as at the higher level of protein intake. At the 7% protein intake level (about 120 mg N daily), the magnesium-deficient ration allowed virtually the same small weight gain as the complete ration, thus confirming for a 10 day period the observations of Frost and Sandy (3). But at the higher level of protein intake (14%)—a moderate one for rat or man—the Mg-deficient ration permitted less than one-fifth the weight gain that the complete ration did. Table I shows the mean weight gain per rat for each group and the amount of basal ration consumed per rat for each group during the 10 day period. Results previously reported(1) for a protein intake level of about 9% are included in the table.

Anorexia appeared in Group 4 (14% protein, Mg-deficient) the fourth day, half of the rats showing marked vasodilatation of the skin of the ears by the sixth day, as first described by McCollum and Orent(4). Group 2 (7% protein, Mg-deficient) showed only a slight but statistically insignificant decrease in food intake and in weight gain.

Comments. Assumptions and limitations of gauging protein synthesis by weight-gain have been noted(1). As far less protein is synthesized on Mg-deficient rations at the 14% level of protein intake than at the 7% level, more Mg is required for normal protein metabolism at the 14% level than at the 7% level.

On a 9% protein intake, a Mg-deficient ration permits more protein synthesis than a complete ration on a 7% protein intake, because lack of protein is the chief limiting factor at the 7% level, enough Mg probably being mobilized from body stores, notably bone, for the slow rate of protein synthesis. On a 9% protein intake, the amount of Mg so mobilized is not sufficient to assure as much protein synthesis as will a complete mineral supplement. The observed results are consistent with the view that at the 9% protein level more Mg is required than at the 7% level. That the worst results are obtained with a Mg-deficient ration at the highest of the 3 protein levels indicates that more Mg is required at the 14% level than at the 7 or

9% levels. If Mg were required merely to form part of the structure of new tissue, at least as much weight would be gained on a 14% protein intake as on a 9% protein intake with Mg-deficient rations.

Comparative studies of mineral supplements, conducted on a 7% protein intake (about 120 mg N daily, the quantity employed by Frost and Sandy(3) in studying Mg-deficiency), may fail to reveal injurious effects of deficient mineral rations, because such effects may only become evident when N-intake is sufficient to assure a normal or optimal rate of protein synthesis.

Summary. Adult protein-depleted rats receiving a complete mineral supplement gain over twice as much in 10 days on a 14% level of protein intake as on a 7% (120 mg N daily) level, but those receiving a Mg-deficient ration gain over twice as much on a 7% level of protein intake as on a 14% level. These results indicate that the Mg requirement for normal protein metabolism increases during protein synthesis as protein intake is increased from 7% to 14% and that the increased Mg requirement is not merely for the structural inclusion of Mg in new tissue. A 7% level of protein intake permits only a markedly subnormal rate of protein synthesis in a previously depleted animal, but 9% and especially 14% levels of protein intake insure more normal rates of protein synthesis and reveal the markedly deleterious effects of magnesium-deficient rations on protein synthesis within 10 days.

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1. Menaker, W., and Kleiner, I. S., Proc. Soc. Exp. Biol. and Med., 1952, v81, 377.

2. Cannon, P. R., Frazier, L. E., and Hughes, R. H., *Metabolism*, 1952, v1, 49.

3. Frost, D. V., and Sandy, H. R., Proc. Soc. Exp. Biol. and Med., 1953, v83, 102.

4. McCollum, E. V., and Orent, E. R., *J. Biol. Chem.*, 1931, v92, xxx (Proceedings).

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Human Body Lice. IV. Direct Serial Passage of Typhus Rickettsiae by Oral Infection.* (20814)

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In connection with projected studies(1) of mutations in microorganisms which propagate in arthropods, methods for performing serial passages are necessary. This paper describes a simple method for serial passage of rickettsiae in lice and presents the results of its application to experimental studies of typhus.

Materials and methods. The origin and maintenance of our normal louse colony has been described(2). When lice were selected for these experiments, they were insects of the third instar, starved for 18 to 24 hours before the infective meal was offered. Experimental infections were initiated by using yolk sac propagated organisms of the Wilmington and Florida(3) strains of *Rickettsia mooseri* and the E strain(4) of *R. prowazeki.* Infection of lice was accomplished by a chicken skin membrane technic(5), and infected lice were fed daily upon normal rabbits(6).

Serial passage. Two or more infected lice

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were examined daily for the presence of rickettsia-like organisms in smears of intestinal tissue stained by Macchiavello's method. When numerous rickettsiae were seen, it was probable that any single louse of the group contained sufficient organisms to act as a donor in passage. Accordingly, 3 or 4 recently emerged adult lice were allowed to cling to the fibers of a sterile cotton swab. Since the integument of recently emerged lice had been exposed for the shortest possible time to bacterial contamination, the use of such insects was preferable. In order to reduce the numbers of surface contaminants, lice were washed by plunging the swab into a 2 ml portion of sterile K7G(7) at room temperature and rotating it rapidly for 1 minute. This procedure was repeated with 10 to 15 fresh portions of sterile liquid. The washed lice were placed in a dry sterile petri dish awaiting dissection. Lice were dissected under aseptic precautions. A single insect was placed in a small drop of K7G(7) on a chilled slide which rested on a covered petri dish containing cracked ice. A small portion of intestinal tissue was teased free and smeared on the surface of the slide, while the remaining intestinal tissue and contents were aspirated through a 23 gauge needle attached to a 1 ml syringe containing diluent. The contents of this syringe were kept chilled.

A prospective donor louse was regarded as suitable if numerous rickettsiae, but no bacteria, were seen in the smear preparation. The contents of the corresponding syringe were ejected into a small chilled tube and aspirated several times in order to macerate the infected tissues. The resulting suspension was mixed with defibrinated or heparinized blood of a normal rabbit. This mixture was then offered by the membrane technic to lice of the next consecutive passage. After recipient lice had fed to repletion, a portion of the meal was often used for injection of animals to detect viable rickettsiae. The remainder of the meal was frozen in an ampoule and stored at –70°C. Surviving lice of a given passage were maintained alive until rickettsiae had been seen in smears of lice of the next passage. At that time, lice of the previous passage were sacrificed, frozen, and stored at –70°C.

TABLE I. Complement Fixation Patterns of Sera of Cotton Rats Inoculated with Viable *Rickettsia mooseri* after Louse Passage.*

Strain	Louse passage	Denominator of highest serum dilution in which complement was fixed by specific antigen Murine	Epidemic
Wilmington	3rd	320	40
”	19th	640	40
Florida	2nd	480	30
”	7th	640	80

* Complement fixation tests were performed according to the method of the Army Medical Service Graduate School(18).

Results. The Wilmington strain of *R. mooseri* was maintained for a total of 95 days through 19 consecutive louse passages. Lice of a given passage were incubated for 3 to 6 days at 32°C before the strain was passed to the next group. The tissues of a single louse were mixed with diluent and blood each time, the total volume of meal offered to recipient lice being 1.2 to 1.3 ml. The dilution thus effected made it unlikely that 19th passage lice contained surviving rickettsiae of the original yolk sac material. Evidence of multiplication of rickettsiae was provided by the massive numbers of organisms in smear preparations.

In an essentially similar manner, the Florida strain of *R. mooseri* was maintained for a total of 43 days through 8 consecutive louse passages. The serologic patterns of cotton rats inoculated with these 2 murine typhus strains after louse passage were typical of *R. mooseri* in the complement fixation reaction (Table I).

The E strain of *R. prowazeki* was maintained for a total of 79 days through 5 louse passages.‡ Although infected lice of the final passage contained rickettsiae which evoked typical serologic patterns in test animals, studies of the ability of the strain to evoke a febrile reaction in guinea pigs were unsatisfactory, owing to the atypical reactions of control animals.

Additional experiments were designed to test the ability of *R. mooseri* to multiply in lice which were nourished on serum instead of blood containing formed elements. Lice which had ingested a 10% suspension in K7G of yolk sac infected with the Wilmington strain were subsequently nourished on single daily meals of Seitz-filtered human serum, which was offered by the membrane technic. Smears of the intestines of these lice provided visual evidence of multiplication of rickettsiae after three days. A second series of lice ingested a meal composed of the intestines of 2 lice of the 7th passage of the Wilmington strain, suspended in a mixture of 0.4 ml K7G and 1 ml serum. During the first 2 days thereafter, the lice fasted at 27°C. On each subsequent day, they were offered meals of normal serum and maintained at 32°C. Visual evidence of multiplication of rickettsiae was provided by smears of lice incubated for 3, 4, and 5 days following ingestion of the infective meal. The cloudy, moth-eaten appearance of the cytoplasm of the intestinal lining cells suggested that the lice were suffering from nutritional deficiency. All lice but one were dead by the 5th day. No change in murine properties of the rickettsiae was demonstrated by the serologic patterns of cotton rats inoculated with a suspension of this 5th day louse. Further passages in lice nourished on serum were not attempted.

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‡ These passages were performed by Peter Skaliy while a student in this Department.

Discussion. By means of the present technic, passage of rickettsiae from donor to recipient lice is accomplished without the interposition of a different infected host species. Rickettsiae are administered by the oral route, as opposed to the method of rectal injection. The only serious technical problem encountered in the present study was the death of recipient lice following ingestion of a meal containing bacterial contaminants from the tissues or surfaces of donor lice. Previous studies of serial passage of rickettsiae in lice have employed the technics of intra-rectal injection(8-14), inoculation into the body cavity of the insect(13,15), or the feeding of lice by a bleb technic(4,16).

It is of interest that no fundamental change in the properties of a strain of rickettsiae has been reported after serial louse passages. The derivation of the E strain from the Madrid strain(4) of *R. prowazeki,* however, demonstrates that the properties of rickettsiae are not inflexible. A further example consists of the changes in virulence of *R. rickettsi* observed during passages in suitable animals (17). Hence the possibility of changes in properties of rickettsiae during passage in arthropods merits further study.

Summary. A simple feeding technic for serial passage of rickettsiae in human body lice is described. Consecutive louse passages are not interrupted by growth of the organisms in other host species. Application of the method to serial passages of epidemic and murine typhus rickettsiae is reported. No changes in properties of the strains were noted during louse passage. The multiplication of murine typhus rickettsiae in lice nourished only on human serum is reported.

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1. Snyder, J. C., *Postgraduate Med.,* 1953, v13, 331.
2. Fuller, H. S., *Am. J. Hyg.,* 1953, v58, 188.
3. Rickard, E. R., *Am. J. Trop. Med.,* 1951, v31, 306.
4. Perez Gallardo, F., and Fox, J. P., *Am. J. Hyg.,* 1948, v48, 6.
5. Fuller, H. S., Murray, E. S., and Snyder, J. C., *Pub. Hlth. Rep.,* 1949, v64, 1287.
6. Snyder, J. C., and Wheeler, C. M., *J. Exp. Med.,* 1945, v82, 1.
7. Bovarnick, M. R., Miller, J. C., and Snyder, J. C., *J. Bact.,* 1950, v59, 509.
8. Weigl, R., *Beitr. z. Klin. d. Infektionskr. u. z. Immunitätsforsch.,* 1920, v8, 353.
9. da Rocha-Lima, H., and Sikora, H., in Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden,* Abt. XII, Teil 1, 1925, 769.
10. Bacot, A., and Segal, J., *Brit. J. Exp. Path,* 1922, v3, 125.
11. Weigl, R., *C. R. Soc. Biol., Soc. Polon. Biol.,* 1930, v103, 823.
12. Weyer, F., *Z. Hyg. u. Infektionskr.,* 1952, v135, 280.
13. ———, *Acta Tropica,* 1952, v9, 170.
14. ———, *Am. J. Trop. Med. Hyg.,* 1952, v1, 809.
15. ———, *Z. Tropenmed. u. Parasitol.,* 1950, v2, 40.
16. Murray, E. S., and Snyder, J. C., *Am. J. Hyg.,* 1951, v53, 22.
17. Price, W. H., *Science,* 1953, v118, 49.
18. Scoville, A. B., Jr., Bennett, B. L., Wertman, K., and Gauld, R. L., *Am. J. Hyg.,* 1948, v47, 166.

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Improved Method for Cultivation of *Brucella* from the Blood. (20815)

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In the diagnosis of *Brucella* infections, particularly in man, positive blood cultures have been recognized as one of the most infallible clinical criteria. Unfortunately, even the most widely adopted method for such blood cultures, namely Castaneda's double medium technic(1) suffers from the disadvantage that cultivation in liquid media cannot be eliminated due to the relative scarcity of organisms in the blood. Such cultivation *in vitro*, even for brief periods, is undesirable because (a) certain lots of a widely used liquid medium, tryptose broth, have proved to contain antibrucellar substances(2), and (b) cultivation in liquid media, particularly in the presence of antiserum, promotes rapid population changes that may lead to the establishment of variant types differing considerably from the type(s) originally present *in vivo*(3). An additional problem associated with blood cultures, namely the removal of inhibitory antibodies and the release of possibly intracellular brucellae by "washing" of the blood specimen, has been investigated by Pickett and Nelson(4) who claim to have obtained a significant increase in positive recoveries following washing of citrated blood with distilled water. However, the latter method also utilized fairly prolonged cultivation in liquid media and has not found widespread acceptance, probably due in part to the authors' controversial claims that they recovered, even from the blood of clinically normal humans, many atypical *Brucella* variants with this technic.

An approach to a more ideal blood culture technic, permitting the recovery of *Brucella* cells directly onto solid media, became possible with the help of membrane filters ("millipore filters"(5)) which are now commercially available.* This new technic has been carried out as follows: One ml of blood is drawn from the heart of an infected rabbit, or from the vein of a patient, and is transferred immediately into a sterile test tube containing 0.1 ml of a heparin solution (100 USP units). Three ml of tryptose saline are used to wash down the sides of the tube and the mixture of heparinized blood and tryptose saline is centrifuged for 30 minutes at 2500 RPM. After the supernate is drawn off, the cell sediment is suspended in 3 ml of sterile distilled water and permitted to stand for a period of 30 minutes. The laked blood sample is then filtered through a sterile membrane filter, employing an O'Leary-Volpe type holder for the filter. Filtration is carried out under negative pressure and usually takes from 30 to 40 minutes. When the entire sample of laked blood has passed through the filter, the disc is removed with the help of sterile forceps and is placed on the surface of a solid medium in Petri dishes (2-1 agar(3) has been employed in all studies so far). If *Brucella* cells are present on the surface of the membrane filter they will develop into visible colonies within 3 to 4 days. The detection of such colonies can be facilitated by the incorporation of 0.5% of 2,3,5 Triphenyltetrazolium chloride into the agar. Colonies may be picked from the surface of the membrane filter and can be subcultured onto appropriate media for further identification.

In order to determine whether this method would permit the quantitative recovery of a known number of *Brucella* cells, a number of experiments were carried out with sterile blood samples to which a known number of *B. suis* cells had been added. One ml samples from a saline suspension containing approximately 10 cells per ml were processed in 3 different ways: (a) direct counts were made on the cell suspensions by the pour plate method, (b) 5 ml of sterile saline was added to each sample and the suspensions were then filtered through membrane filters which were subsequently placed on the surface of 2-1 agar plates, and (c) samples were added to heparinized blood obtained from normal rabbits

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* Obtainable from Lovell Chemical Co., Watertown, Mass.

TABLE I. Recovery of *Brucella suis* Cells from Infected Rabbits by Membrane Filter Method and by Castaneda Method at Successive Days after Infection.

Days after inoculation	A* Membrane filter	A* Castaneda	B Membrane filter	B Castaneda	C Membrane filter	C Castaneda	D Membrane filter	D Castaneda
1	3	+	12	+	0	—	1	—
3	179	+	100	+	54	+	69	+
5	21	+	34	+	3	+	Not run	+
7	2	+	10	+	2	—	243	+
9	5	+	6	+	0	—	8	+
11	24	+	2	—	1	—	1	—
13	0	—	1	—	0	—	0	—
15	0	—	2	—	0	—	1	—
17	0	—	0	—	0	—	0	—
19†	0	—	1	—	2	+	0	—

* Capital letters represent different animals. Figures are numbers of colonies found on membrane filter discs. Positive recoveries by Castaneda method are recorded as plus, non-recovery as minus.

† No positive recoveries from the 21st to the 27th day.

and processed by the above described technic. In 6 replicate tests for each procedure the average recovery of cells from these one ml samples was (a) 11 cells by pour plate method, (b) 13 cells after filtration of the saline suspensions through membrane filters, and (c) 10 cells after filtration of treated blood suspensions through membrane filters. These results indicate that the new technic permits quantitative recovery of *Brucella* cells, at least when they are present extracellularly.

Numerous tests have been made on supernates obtained following centrifugation of laked blood specimens, but *Brucella* cells have never been recovered from such supernates; therefore, all supernates are now discarded in the regular routine.

Finally, comparative tests have been made on the recovery of *Brucella* cells from the blood of infected rabbits at various times after exposure of the animals to *B. suis* employing both the Castaneda method and the above described membrane filter technic. Six rabbits were inoculated intravenously with approximately 1 x 10^7 organisms. On the following day and every other day thereafter, for a period of 27 days, a 2-ml sample of blood was taken from the heart of each of the infected rabbits. One ml of blood was then processed according to the membrane filter technic and the other one ml of heparinized blood was treated according to the method described by Castaneda(1). The results of these tests are shown in Table I and indicate that the recoveries obtained with the help of the new method are as good, and probably better, than those obtainable by previously utilized methods. In addition to the ability to culture *Brucella* cells directly onto solid medium without any growth period in liquid media, this method also appears to permit a quantitative estimation of the extent of bacteremia. It is probable that the same technic may be applicable to blood cultures of other bacterial pathogens.

Summary. A new technic for the recovery of *Brucella* cells from the blood of infected hosts is described. This technic employs filtration of laked blood specimens through membrane filters and permits the recovery of *Brucella* cells directly on solid media. The new method has proved to be at least as good as previously described blood culture methods and does not possess the disadvantage of cultivation in liquid media which is necessary in other methods. The membrane filter technic also appears to permit a quantitative estimation of the extent of bacteremia.

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1. Castaneda, M. R., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 114.

2. Schuhardt, V. T., Rode, L. J., Foster, J. W., and Oglesby, G., *J. Bact.*, 1949, v57, 1.

3. Braun, W., in *Brucellosis,* published 1950, by AAAS.

4. Pickett, M., and Nelson, E. L., *J. Bact.*, 1951, v61, 229.

5. Clark, H. F., Goldreich, E. E., Jeter, H. L., and Kabler, P. W., *Pub. Health Repts.*, 1951, v66, 951.

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Ultraviolet Absorption Spectrum of Protyrosinase and Tyrosinase.* (20816)

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In a previous communication it was shown that no change in the sulfhydryl or disulfide groups of the protyrosinase molecule seems to occur upon activation and conversion to the active form, tyrosinase(1). In the present series of experiments the ultraviolet absorption spectrum of protyrosinase and tyrosinase was determined to ascertain whether a difference exists between the inactive and active forms of the enzyme. Manometric measurements of the enzymatic properties of these two substances show a definite difference with respect to the oxidation of tyramine HCl(2). The ultraviolet absorption spectrum should demonstrate a structural change if such exists.

In a study of the physical or chemical properties of a substance, a primary requirement is that one knows the "purity" of his preparation. During a series of extractions of the egg of the grasshopper, (*Melanoplus differentialis*), we obtained a protyrosinase which was considerably more concentrated than the original extractions. As we fractionated our preparation, the amount of non-dialysable substances decreased, the rate per mg (measured, of course, by activating this proenzyme) increased. The final preparation of the protyrosinase had 0.21% copper. Kubowitz(3) found 0.18% copper in his preparation of tyrosinase; Keilin and Mann (4), 0.3%, and Nelson and Dawson(5), 0.21%. The procedures followed in purifying and identifying the different samples of protyrosinase are similar to those previously described.‡ The preparation used in the present absorption experiment is that designated as B-18a. In the samples whose spectra are graphed for reproduction (Fig. 1) the method of treatment was as follows:

Left side of graph (Fig. 1). The B-18a was taken up in 0.1 M phosphate (pH 6.8) rather than 0.9% NaCl. Two cc of this were diluted to 32 cc with the final dialysate (0.1 M phosphate) and then filtered through a fritted glass filter. Of this, 14 cc were used as proenzyme; 10 cc were activated with 0.5 cc of sodium dodecyl sulfate or with 0.1 cc aerosol OT. The control cell for the proenzyme was the dialysate; for the enzyme the dialysate plus the activator used. These were mixed in the evening, the spectrum determined on the following morning and the activity in the afternoon of that day. The activity of proenzymes plus activator was determined for each protyrosinase solution used.

Right side of graph (Fig. 1). Treated in an identical manner save that the solvent was 0.9% NaCl.

A Hilger quartz spectrophotometer with a hydrogen arc light source was used.‡ An examination of Fig. 1 shows a distinct difference in the absorption spectra of the active and inactive form of the enzyme between 230 and 260 mμ. An absorption maximum at 310 mμ is common to both and has been attributed to the copper present by Dalton and Nelson(6). The data cannot be specifically interpreted. The increase is in the range of absorption of the ring structure amino acids. Theoretically, the absorption of the amino acids should be the same and in relation to the amounts and kinds present (this could not vary in these experiments). Lavin, Loring, and Stanley(7) have shown that structure may influence the absorption of the amino acids. Further work must be done on the absorption below 270 mμ to distinguish the various absorption bands of individual amino acids. Removal of the copper should change the absorption at 310 mμ if the assumption that this is due to copper is correct.

Summary. We have purified to some extent the proenzyme (protyrosinase) without activating it. There is a change in the absorption

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* Aided by a grant from the National Institutes of Health.

† Now in Department of Physiology, University of Washington, Seattle.

‡ We are indebted to the Department of Physical Chemistry for use of this apparatus.

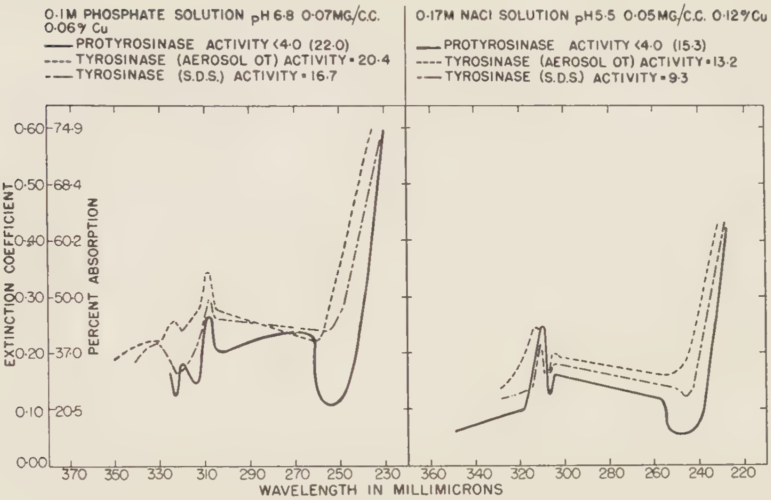

FIG. 1. Shows ultraviolet absorption curves for preparation, B-18a, both as protyrosinase and tyrosinase. Rates expressed as 4 for protyrosinase samples are impossible to accurately measure and can be assumed to represent zero activity. All other rates shown are for tyrosinase or activated protyrosinase. For further description see text.

spectrum in the ultraviolet accompanying the activation of the protyrosinase molecule to the active tyrosinase form.

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1. Bodine, J. H., and Carlson, L. D., Proc. Soc. Exp. Biol. and Med., 1953, v83, 717.

2. Allen, T. H., and Bodine, J. H., *Proc. Nat. Acad. Sc.*, 1941, v27, 269.

3. Kubowitz, F., *Biochem. Z.*, 1937, v292, 221.

4. Keilin, D., and Mann, T., *Proc. Roy. Soc. London*, 1938, B125, 187.

5. Nelson, J. M., and Dawson, C. R., *Tyrosinase, Advances in Enzymology*, 1944, v4, 99.

6. Dalton, H. R., and Nelson, J. M., *J. Am. Chem. Soc.*, 1939, v61, 2946.

7. Lavin, G. I., Loring, H. S., and Stanley, W. M., *J. Biol. Chem.*, 1939, v130, 25.

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Effect of Iodine on Serological Specificity of Tobacco Mosaic Virus.* (20817)

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In complement fixation tests, Weaver and Price(14) found the Type strain of tobacco mosaic virus (TMV) to react optimally with its homologous antiserum, or with antiserum to closely related strains, when a specific weight of virus, designated as the optimum antigenic unit (OAU), was present; TMV strains were differentiated from one another by differences in their OAU as well as by differences in antiserum dilution end points (ADE) in homologous and heterologous reactions, thus indicating a high degree of specificity for the serological reaction used. In precipitin tests, iodinated globulin was found to react weakly or not at all with antiserum to native protein(6,11,16); serum globulin iodinated at pH 7.3 reacted with antiserum to native globulin whereas that iodinated at pH 8.5 did not(6). In the work here reported, an attempt was made to iodinate specific amino acid groups on the TMV particle at neutral (7.0) and alkaline (7.65) pH values and to correlate the changes thus produced with the changes in serological activity.

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* Based on thesis by J. J. B. in partial fulfillment of requirements for Master of Science degree.

TABLE I. Optimum Antigenic Units (OAU) and Antiserum Dilution End Points (ADE) Obtained in Titrating SH Abolished, S-S Abolished, and Diiodotyrosine Virus against Antiserum to Native TMV and Antiserum to Diiodotyrosine Virus.

Antigen	Antiserum to Native TMV* OAU	Antiserum to Native TMV* ADE‡	Antiserum to Diiodotyrosine TMV† OAU	Antiserum to Diiodotyrosine TMV† ADE‡
	μg		μg	
Native virus	2.8	320	2.8	40
SH abolished virus	2.8	320	2.8	40
S-S " "	5.6	160	5.6	40
Diiodotyrosine "	5.6	10	5.6	20

* Results of 4 titrations.
† " " 3 " .
‡ Reciprocals.

Materials and methods. Antigens and antigen preparation. The Type strain of TMV was grown in Turkish tobacco plants and then concentrated and purified by alternating cycles of low (2950 x G for ½ hour) and high (25000 x G for 2 hours) speed centrifugation. The pellets obtained were suspended in 0.85% saline and stored at –20°C. *Antiserum to native and iodinated TMV.* Anti-TMV serum was prepared by injecting rabbits intraperitoneally with 2-ml portions of purified virus solutions containing about 26.5 mg/ml on the first and third day of initial immunization. Thereafter, one ml portions were administered intraperitoneally or intravenously every fourth day for 35 days. Preparation of antiserum to diiodotyrosine virus differed from this only in the volumes administered initially and the route of injection, 1.0 ml and 0.25 ml portions containing 4 mg/ml being injected exclusively by the intraperitoneal route. All sera were inactivated at 56°C for ½ hour. *Iodine-treated virus.* Abolition of sulfhydryl (SH) groups was accomplished with various volumes of 0.05 N iodine in 0.18 N potassium iodide. The preparation was maintained at pH 7.0 with phosphate buffer and the reaction allowed to proceed for 10 minutes at 35°C. The reaction was usually complete in 10 minutes as indicated by a negative nitroprusside test and the disappearance of iodine by a negative starch test(2). The reaction was reversible when 2 N KCN in the presence of 27% ammonium hydroxide was added. To accomplish the oxidation of SH groups past the S-S stage, quantities ranging from 0.05 to 0.08 ml of 0.05 N I-KI were used. Treatment for 15 to 25 minutes at 35°C was required to oxidize completely all SH groups past the S-S stage at pH 7.0. pH changes were no greater than 0.2 pH unit on the acid side of neutrality after reaction. Negative cyanide nitroprusside tests(1) indicated the absence of the S-S linkage. Insoluble protein was removed by centrifugation. Diiodotyrosine virus was prepared in the manner described by Anson and Stanley(2). Iodination was carried out at pH 7.65 in the presence of 0.09 N I-KI. The reaction was allowed to proceed for one hour at 56°C. All resulting preparations were tyrosine negative. Insoluble protein was removed by centrifugation. Excess iodine was neutralized with N/10 sodium thiosulfate. Millon tests performed on the supernatant indicated that tyrosine had been converted to diiodotyrosine. The preparation was dialyzed for 27 hours against flowing distilled water to remove tetrathionate and to readjust the pH to approximately 6.9. The preparation was centrifuged and the concentration of remaining protein determined by the micro-Kjeldahl method of Ma and Zuazaga(9). *Complement fixation technic.* The complement fixation technic used has been described in detail by Wertman(15) and by Tall *et al.*(12). ADE and OAU were determined from grid titrations involving 2-fold serial dilutions of antigen and 2-fold serial dilutions of antiserum(14). The OAU has been defined as the weight of virus per ml in the highest dilution of antigen giving a 4+ reaction with the highest dilution of antibody, the latter being defined as the ADE.

Results. On the basis of past experience, 2-fold differences in OAU are significant but 2-fold differences in ADE have doubtful validity unless based on a large number of titrations(14). The 2-fold differences in ADE (Table I) are therefore questionable, whereas 4-fold differences are undoubtedly significant.

The OAU for native TMV, 2.8 μg, was the same as that found by Weaver and Price(14). Virus with SH groups abolished gave exactly the same reactions with both antisera as the native virus, indicating that neither cysteine nor cystine, as such, plays a major role in serological specificity. Abolition of disulfide groups, however, resulted in increasing the OAU by 2-fold and, in the reaction with antiserum to the native virus, decreasing the ADE by 2-fold. The latter is questionable but there is a possibility that it may have resulted from an effect of iodination on tyrosine groups; although Millon tests on S-S abolished virus were strongly positive, this does not exclude the formation of monoiodotyrosine.

The most significant changes occurred with the diiodotyrosine virus; in reactions of antiserum to native TMV with diiodotyrosine virus the OAU was doubled and the ADE reduced 32-fold as compared to the homologous reaction with native virus. This suggests the importance of aromatic groups for combination of antigen and antibody and agrees in principle with observations of others(6,16). Increase in OAU can probably be explained on the basis of a shift in redox potential of iodine, since a shift of 1.53 pH units on the acid side of neutrality was measured(3).

Reactions between the various antigens and antiserum to diiodotyrosine virus are of particular importance because they show that the treated virus was still capable of stimulating antibody formation in rabbits, despite the comparatively low titer. Because of the low titer it is virtually certain that completely negative results would have been obtained by the precipitin technic, as has indeed been the experience of others(6,11,16). The sensitive complement fixation test thus demonstrated serological activity of chemically altered virus not demonstrable by the relatively insensitive precipitin test.

Discussion. Although many postulations for the intermediate reaction between cysteine and cystine and oxidants are found in the literature(4,5,13) none offers proof of the exact intermediate with chemically treated proteins. Evidence that RSO_3H could not be formed in the reaction between iodine and cysteine was given by Anson and Stanley(2).

It is theorized that TMV contains sub-antigenic groupings, as well as the known aromatic determinants; change in sub-antigenic groupings, such as oxidation past the S-S stage, leads to a change in the OAU, whereas change in antigenic groupings, of which tyrosine is representative, leads to drastic change in the ADE.

Moorhead and Price(10) found that antiserum to the Type strain of TMV reacted with the Rosette strain of TMV with an ADE 8-fold less than that in the homologous reaction, whereas antiserum to the Rosette strain reacted with the Type strain antigen at the same ADE as in the homologous reaction. Kleczkowski(7) reported a similar relationship between the Type and Aucuba strains of TMV and their respective antisera when the quantitative precipitin test was used. A similar result can be seen in Table I with normal and diiodotyrosine virus. Knight(8) found no difference in tyrosine content between the Type and Aucuba strains of TMV which could account for the serological differences observed.

Summary. The sensitive complement fixation reaction was used to detect antigenic differences between normal tobacco mosaic virus (TMV) and TMV treated with iodine in KI in such a manner as to oxidize SH groups or to form diiodotyrosine. No change in antigenicity was detected in TMV in which the SH groups had been oxidized to S-S. Oxidation past the S-S stage resulted in change in the weight of virus reacting optimally with antiserum. Virus in which tyrosine was converted to diiodotyrosine was only weakly effective in stimulating formation of antibodies in rabbits and reacted with antiserum to normal virus with a 32-fold drop in antiserum dilution end point. These serological differences are comparable to the serological differences occurring between strains of TMV.

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1. Anson, M. L., *J. Gen. Physiol.*, 1940-41, v24, 399.

2. Anson, M. L., and Stanley, W. M., *ibid.*, 1940-41, v24, 679.

3. Herriott, R. M., *Advances in protein chemistry*, Acad. Press, Inc., N. Y., 1947, v3, 169, (p207).

4. Hughes, W. L., and Straessle, R., *J. Am. Chem. Soc.*, 1950, v721, 452.

5. Kassel, Beatrice, and Brand, E., *J. Biol. Chem*, 1938, v125, 131.

6. Kleczkowski, A., *Brit. J. Exp. Path.*, 1940, v21, 98.

7. ———, *ibid.*, 1941, v22, 44.

8. Knight, C. A., *J. Biol. Chem.*, 1947, v171, 297.

9. Ma, T. S., and Zuazaga, G., *Ind. and Eng. Chem.*, 1942, v14, 280.

10. Moorhead, E., and Price, W. C., *Phytopathology*, 1953, v43, 73.

11. Obermayer, F. R., and Pick, E. P., *Wien. Klin. Woch.*, 1906, v19, 327.

12. Tall, M. G., Price, W. C., and Wertman, K., *Phytopathology*, 1949, v39, 288.

13. Toennis, G., *J. Biol. Chem.*, 1938, v127, 27.

14. Weaver, E. P., and Price, W. C., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 125.

15. Wertman, K. F., Doctoral Dissertation, Univ. of Pittsburgh, 1947.

16. Wormall, A., *J. Exp. Med.*, 1930, v51, 295.

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Partially Depolymerized Hyaluronic Acid (PDHA) as a Spreading Agent. (20818)

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The spreading action of testicular hyaluronidase is usually attributed to the decrease in viscosity of the ground substance which results from depolymerization of the hyaluronate by the enzyme. The effect is to decrease the normal resistance of the barrier. A more recent view suggests that the enzyme releases PDHA which as a protective colloid and peptizing agent participates actively in the dispersion and transfer of materials through the ground substance and in the urine(1). It has also been suggested that hyaluronic acid is capable of acting as an ion exchange resin(2). In this paper we present data on the spreading properties of PDHA prepared from *in vitro* hydrolysis of streptococcal hyaluronic acid by testicular hyaluronidase.

Materials and methods. Bacterial hyaluronic acid was isolated by Dr. George H. Warren from a virulent strain of group A *Streptococcus pyogenes* obtained from the Hospital of the Rockefeller Institute. It contained 3.67% N and was free from S, P, and halogen. Purified bovine vitreous humor hyaluronate and human umbilical cord hyaluronate were prepared by Dr. Harvey E. Alburn. Streptococcal hyaluronate before treatment with the enzyme was the most viscous and vitreous humor hyaluronate the least viscous preparation. PDHA was prepared by incubating streptococcal hyaluronic acid with testicular hyaluronidase* for 15 minutes at 38°C. The incubation mixture was autoclaved for forty minutes at 104°C and the resulting precipitate removed by centrifugation and discarded. The supernatant was found to be free from hyaluronidase by the turbidimetric assay and therefore contained less than 0.1 TRU/ml. Incubation of the substrate with the enzyme for one hour resulted in supernatants that had no significant spreading activity. Spreading tests were made on albino rabbits of both sexes weighing between 2.3 kg and 2.9 kg. The intradermal injections were made through a 26 gauge, one-half inch needle into the lumbar and scapular regions clipped free from hair. The injections consisted of 0.1 ml of 1% trypan blue in physiological salt solution (PSS) mixed with 0.1 ml of test solution. Measurements of the large diameter of spread (D) and the small diameter of spread (d) were made with vernier caliper immediately after injection and 15, 30, and 60 minutes later. The area of spread was computed from the formula for the area of an ellipse, $A_{\text{sq mm}} = \frac{D \times d \times \pi}{4}$. Each concentration of a substance was tested on a minimum of 6 rabbits. Each rabbit received a control injection of PSS. The injection sites for the control and materials to be tested were randomized. The comparative efficiency of hyaluronidase and PDHA to facilitate hypodermoclysis of PSS was carried out in rabbits. Six rabbits received

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* Wydase®—Wyeth Laboratories, Inc.—7000TRU/mg N.

TABLE I. Effect of Increasing Concentrations of Hyaluronidase on the Spreading Reaction.

	Area of spread in 60 min. (mm²)					
TRU inj.	100	1000	2000	5000	10000	20000
Mean	197.2	388.2	483.0	643.6	675.6	587.2
± SD %	9.2	4.8	10.6	4.4	2.8	5.4
No. of rabbits	6	8	6	10	6	6

100 ml of PSS in one flank and 100 ml of PSS containing 150 TRU of hyaluronidase in the other. Six rabbits received 100 ml of PSS in one flank and 100 ml of PSS plus 3 ml of 5% PDHA in the other. A comparison of the ability of hyaluronidase and PDHA to facilitate the spread and absorption of an x-ray contrast medium was made in female guinea pigs. Eight guinea pigs received 1 ml of Urokon® sodium† (Mallinckrodt) mixed with 3 ml of PSS subcutaneously over the lumbar region. Eight guinea pigs received 1 ml of the contrast medium plus 3 ml of PSS containing 150 TRU of hyaluronidase. Eight guinea pigs received 1 ml of the contrast medium plus 3 ml of 5% PDHA. Serial x-rays were taken at 5 minute intervals and the area of spread of the contrast medium was measured with a planimeter. The data were analyzed statistically for homogeneity within groups and for significant differences between groups by analysis of variance for paired and unpaired data.

Results. The mean area of spread in the 78 control injections consisting of PSS and trypan blue was 153.5 sq mm with a standard error of ± 5.25%. The effect of increasing concentrations of hyaluronidase on the area of spread is shown in Table I. It will be seen that increasing the concentration of enzyme from 0 to 10000 TRU per injection resulted in increasing area of spread. Further increase to 20000 TRU resulted in a sharp decline in spreading activity. It was not possible to inject larger amounts of the enzyme because of the viscosity of the solution. Plotting the logarithm of the turbidity reducing units against the mean area of spread results in a straight line with a slope of 2.34 from 100 to 10000 TRU.

Spreading effects of hyaluronates from different sources. Streptococcal, umbilical cord, and vitreous humor sodium hyaluronates had no practical spreading action although statistically each of the substances exhibited some spreading at one concentration and an inhibiting effect at a higher concentration. Tested in groups of 6 to 8 rabbits in concentrations of 50, 100, 1000, 2000, and 5000 μg of sodium hyaluronate the streptococcal preparation gave respectively means (± SD%) of 147.1 (7.8), 217.2 (14.4), 198.1 (9.3), 163.7 (15.9), and 100.3 (20.3) sq mm of spread in 60 minutes. The umbilical cord preparation gave respectively means of 160.7 (8.5), 193.5 (5.6), 202.2 (6.6), 149.5 (5.2), and 112.9 (6.9) sq mm of spread in 60 minutes. The vitreous humor preparation gave respectively means of 202.2 (6.8), 194.7 (15.1), 171.5 (12.2), 142.3 (11.8), and 138.3 (4.1) sq mm of spread in 60 minutes. The spreading action was not inversely related to viscosity of the preparation. Streptococcal hyaluronate which gave the most viscous solutions had the greatest spreading action whereas the vitreous humor hyaluronate which gave the least viscous solutions had the least spreading action. Further evidence that the viscosity alone was not the determinant in the spreading action is seen from the fact that the lowest concentration was not always the most effective spreading concentration. Other polymers such as degraded alginic acid, undegraded pectin, and clinical grade dextran tested in identical fashion and in identical concentrations showed no spreading activity statistically.

Spreading action of PDHA. From Table II it will be seen that PDHA had considerable spreading activity which almost equalled that produced by 2000 TRU of hyaluronidase. It will also be seen that more completely hydro-

TABLE II. Effect of Depolymerized Sodium Hyaluronate (Streptococcal) on the Spreading Reaction.

	Area of spread in 60 min. (mm²)	
	Enzymatic hydrolysis	
	15 min.	1 hr
	5 mg inj.	5 mg inj.
No. of rabbits	12	6
Mean	553.1	241.3
± SD %	10.6	12.4

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† Sodium Acetrizoate 30% and 0.1% sodium citrate.

TABLE III. Effect of Hyaluronidase and PDHA on Hypodermoclysis in Rabbits. 6 rabbits in each series.

	Time for hypodermoclysis (min.)			
	Saline	Hyaluronidase (150 TRU)	Saline	PDHA (150 mg)
Mean	47	19	51	18
± SD %	7.6	10.2	8.4	12.7

TABLE IV. Effect of Hyaluronidase and PDHA on Absorption and Excretion of Urokon.

Treatment group*	Spread, mm² at time of inj.		Appearance in urinary bladder (min.)
Saline + Urokon	Mean	4.4	30
	± SD %	22.7	
150 TRU hyaluronidase + Urokon	Mean	10.4	16.3
	± SD %	16.3	
150 mg PDHA + Urokon	Mean	11.4	16.9
	± SD %	30.7	

* 8 guinea pigs/group.

lyzed hyaluronate was without significant spreading activity.

Hypodermoclysis and absorption of x-ray contrast medium. The ability of PDHA to facilitate a hypodermoclysis of PSS in rabbits is shown in Table III. It will be seen that 150 mg of PDHA was as effective as 150 TRU of the enzyme. In other experiments 50 and 100 mg of PDHA added to the clysis was without facilitating effect. Facilitation of spread and absorption of an x-ray contrast medium in guinea pigs is shown in Table IV. It can be seen that 150 TRU of hyaluronidase or 150 mg of PDHA increased the area of spread and the rate of absorption and appearance of the opaque material in the urinary bladder by twofold.

Summary. 1. Increasing the concentration of hyaluronidase by large units resulted in increased spreading activity until a massive concentration of enzyme was used. 2. Highly polymerized hyaluronic acid obtained from *Streptococcus pyogenes,* bovine vitreous humor, and human umbilical cord had no significant spreading action. 3. Partially depolymerized streptococcal hyaluronic acid had significant spreading action in rabbits, hastened the absorption of a clysis of physiological salt solution in rabbits and facilitated the spread and absorption of an x-ray contrast medium in guinea pigs. 4. Further depolymerization of the streptococcal hyaluronic acid resulted in products that no longer had significant spreading action.

We wish to acknowledge the technical assistance of Mr. Edward E. Livingston, Jr.

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1. Seifter, J., *Bull. schweiz. Akad. med. Wiss.,* 1952, v8, 67.

2. Meyer, K., and Rapport, M., *Science,* 1951, v113, 596.

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Topical Protection Against Hyperergic Inflammations by a Preceding Local Inflammation. (20819)

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The hyperergic reaction provoked in the rat by the intraperitoneal injection of fresh egg albumin(1) can be topically inhibited by means of a previously produced local inflammation. This was demonstrated by Selye and his collaborators(2), who stated that the degree of topical protection depends on the intensity of the preceding inflammation and considered the phenomenon as not specific, since various irritative substances inhibit topically the hyperergic reaction produced by the egg albumin injection. As regards the mechanism of this protecting action, the aforementioned authors assert that it is strictly locally conditioned.

Method. Fifty white 4-months-old rats were used, with a body weight of between 100 and 130 g, distributed in 5 groups of 10 rats each. All rats received in their right leg a subcutaneous injection of 0.25 ml of a 4% solution of peptone in water. The rats of the *first* group were given 24 hours later an intraperitoneal injection of 0.5 ml of a 50% solution of recently obtained egg albumin. The *second* group had the right sciatic nerve severed 20 hours after the peptone injection in their right leg, and 4 hours later received an intraperitoneal injection of 0.5 ml of the albumin solution. The animals of the *third* group were adrenalectomized 18 hours after the first injection, and 6 hours later 0.5 ml of egg albumin was injected. The rats of the *fourth* group were adrenalectomized 6 hours before the peptone injection in their right leg, and 24 hours later they received an intraperitoneal injection of 0.5 ml of albumin. Finally, in those of the *fifth* group the suprarenal glands were removed 6 hours before the first injection. One hour after the peptone injection in their leg they received 5 mg of Merck's cortisone acetate intraperitoneally, and 24 hours later an intraperitoneal injection of 0.5 ml of egg albumin.

The surgical operations—sciatic nerve extirpation and adrenalectomy—were carried through with ether anesthesia and under aseptic conditions. Adrenalectomy was performed by the posterior route, and the suprarenal glands as well as the immediate fat were extirpated. After operation the animals received 1% solution of sodium chloride, but otherwise their nourishment was similar to that of the remaining rats. The animals were observed during a period of 4 hours following the albumin injection, the intensity of the general reaction and the swelling of the injected leg being recorded.

Results. The rats of the *first* group showed within 2 hours following the intraperitoneal albumin injection an intense edema and hyperemia of snout and fore-legs; the left leg was edematous and presented a marked hyperemia, whereas the right leg, which had received the peptone injection, showed no reaction at all (Fig. 1, No. 1). The local protection was evident in 9 rats.

Six of the *second* group of rats, whose right sciatic nerve was cut after the local peptone injection, did not show the protection phenomenon, and in the remaining animals this phenomenon was incomplete, the injected leg presenting a slight edema (Fig. 1, No. 2).

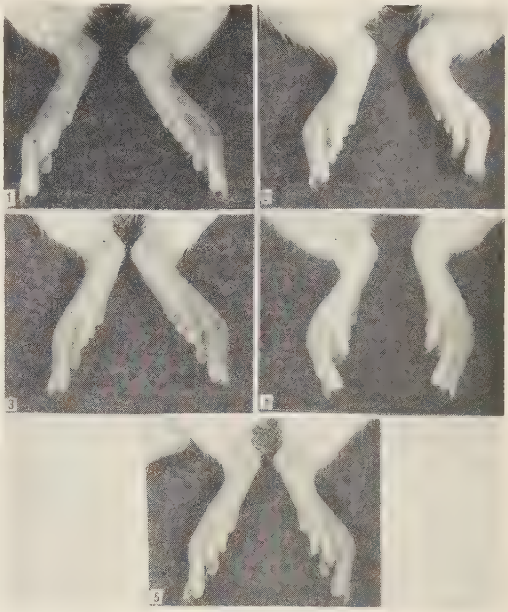

FIG. 1. 1) Injection of 0.25 ml of 4% peptone in right leg. General hyperergic reaction 24 hr later by intraperitoneal injection of 0.5 ml of 50% egg albumin. Right leg protected. 2) Injection of 0.25 ml of peptone in right leg. 20 hr later section of right sciatic nerve, and 4 hr later general reaction by intraperitoneal injection of albumin (0.5 ml at 50%). No protecting effect. 3) 18 hr after the subcutaneous injection of peptone in right leg (0.25 ml) bilateral extirpation of suprarenal glands and 6 hr later peritoneal injection of 0.5 ml of egg albumin. Right leg protected. 4) Bilateral adrenalectomy 6 hr before injection of 0.25 ml of peptone in right leg. 24 hr later general reaction provoked by injection of 0.5 ml of albumin. Absence of the local protecting effect. 5) 6 hr prior to the local peptone injection, bilateral adrenalectomy. One hr after subcutaneous injection of 0.25 ml of peptone in right leg, peritoneal injection of 5 mg of Merck's cortisone acetate. 24 hr later injection of egg albumin (0.5 ml intraperitoneally) to provoke general hyperergic reaction. Right leg protected.

The rats in the *third* group—those adrenalectomized 18 hours following the injection of the locally irritating substance—a phenomenon of local protection against the general reaction provoked by the egg albumin was to be seen (Fig. 1, No. 3).

The animals of the *fourth* group, the suprarenal glands of which were extirpated a few hours prior to the subcutaneous peptone injection in the right leg, did not show the local protection. The protecting effect was lacking in 9 rats (Fig. 1, No. 4).

In 7 of the *fifth* group rats the protection was quite evident, but in the 3 remaining animals the general reaction which usually is produced by the albumin injection did not appear (Fig. 1, No. 5).

Discussion. The results confirm the protecting effect which the preceding local application of an irritative substance is able to produce against the general hyperergic inflammation provoked by an albumin injection, provided that its action precedes the injection of the antigen by an adequate time interval. In the second place, they show that this topical protection phenomenon may be modified by local and general influences, the last of which depend on the suprarenal glands.

The second of the sciatic nerve of the leg into which peptone was injected reduces or suppresses the protecting effect. Possibly this action is connected with the circulatory changes—hyperemia, increase of the capillary permeability—that follow the section of the nerve.

The effects obtained on the animals belonging to the third, fourth, and fifth groups are more interesting, as they suggest that the mechanism of the phenomenon in question may not be strictly local and may possibly, at least in part, depend on general influences, or more concretely, on the action of the suprarenal glands.

While the rats of the third group, which retained their suprarenal glands, during the period of the local inflammation following the subcutaneous peptone injection, display a very clear topical protection phenomenon, in those belonging to the fourth group, the suprarenal glands of which were extirpated prior to the local application of the irritative substance, the protection is lacking or is very limited. The topical protection reappears in the rats without suprarenal glands provided they receive, a short time after the peptone injection in their right leg, an injection of cortisone.

These experiments appear to establish an interrelation between the presence or absence of the local protection phenomenon and the suprarenal gland or its cortical steroids.

The antiphlogistic action of cortisone is well known, and its effect is partly due to the drug's action on the capillary permeability. Moon and Tershavovec(3) showed that the inflammation secondary to the local application of thermic irritating agents is diminished by treatment with cortisone, which provoked an increase of the capillary resistance, and that in these animals the diapedesis, the capillary hemorrhage and the edema are less frequent. Likewise Benditt(4) reported that cortisone diminishes the capillary permeability and reduces the diffusion area which follows the injection of hyaluronidase. Menkin(5) observed that the extract of the suprarenal cortex suppresses the action of leukotoxine or of the total extract of an inflammatory exudate. Later on, the same author(6) found that some commercial preparations of cortisone increased the capillary permeability. It may be that this paradoxical effect is not directly caused by the cortisone, but by the vehicle in which it is suspended, since the powder of this substance does not produce any increase of the capillary permeability.

Apart from this action on the capillary walls, cortisone diminishes the processes of cellular activity, as has been proved repeatedly. Landauner(7) and Karnofsky and his collaborators(8) demonstrated the inhibiting action of cortisone on the embryo's development. More recently, Menkin(9) has shown that cortisone acetate suppresses to some extent the normal segmentation of the fecundated eggs of *Arbatia punctulata*, the sperm also becoming inactive. The same results were obtained by Rodriguez and the author when studying the effect of cortisone upon the germination and development of some seeds (10).

The conditions of the inflammatory focus are modified considerably in all its stages by the action of these steroids. Michael and Whorton(11) demonstrated that the leukocytic infiltration as well as the local fibrin formation and the degree of edema are modified by cortisone during the acute stage of the inflammation. Likewise, Selye(12) observed by means of the pouch granuloma method that the introduction of small quantities of hydrocortisone directly in the wall of the bursa diminishes or prevents the appearing of inflammatory alterations.

In view of these results it appears that the subcutaneous injection of peptone and the secondary inflammation produced by it determine the appearance of an alarm reaction. The steroids of suprarenal source which are produced during this reaction may move from the blood to the inflamed tissues due to the increase of capillary permeability which takes place in the inflamed area, accumulating there and modifying the tissue's conditions and the degree of resistance of the capillary wall; they also may reduce the activity of the tissue's cells and perhaps prevent the appearance of some metabolic factor which is important in the development of the inflammatory process. Menkin(13) has proved this with the preparation F, which prevents or inactivates the formation of leukotoxine and of the L.P.F., so that later on, when the second injection is given, the reaction between the antigen—the egg albumin—and the tissue's cell is prevented or rendered difficult, either because the movement of the antigen across the capillary wall is impeded, or because the cellular reactivity is diminished, which might prevent the antigen's reaction with the cell or might render impossible the local production of antibodies. In any case, the result would be an antiphlogistic action depending for the most part on the stimulation of the suprarenal gland.

Summary. 1. The hyperergic reaction provoked in the rat by the intraperitoneal injection of egg albumin can be topically inhibited by a previously produced local inflammation. 2. The topical protection phenomenon can be modified by local and general influences. The section of the sciatic nerve of the injected leg prevents or diminishes the protecting effect. The extirpation of the suprarenal glands prior to the application of the locally irritating substance prevents the topical protection phenomenon, which reappears if the animals receive immediately after the action of the locally irritative agent an intraperitoneal injection of cortisone. 3. The topical protection phenomenon depends partly on the moving, from the blood to the tissues, of the corticosteroids which are freed during the alarm reaction following the local inflammatory irritation; these corticosteroids have an anti-inflammatory action which diminishes or prevents the development of phlogistic alterations in the tissues which previously have suffered an inflammation.

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1. Selye, H., *Endocrinol.,* 1937, v21, 169.

2. Hoene, R., Labbe, P., Selye, H., Proc. Soc. Exp. Biol. and Med., 1952, v79, 65.

3. Moon, W. H., and Tershakovec, G. A., *ibid.,* 1952, v79, 63.

4. Benditt, E. P., *et al., ibid.,* 1950, v75, 782.

5. Menkin, V., *Am. J. Physiol.,* 1940, v129, 691.

6. ———, *ibid.,* 1951, v164, 294.

7. Landauner, W., *Endocrinol.,* 1947, v41, 489.

8. Karnofsky, D. A., Stock, C., Rhoads, E. P., *Fed. Proc.,* 1950, v9, 290.

9. Menkin, V., Proc. Soc. Exp. Biol. and Med., 1953, v82, 189.

10. Rodriguez, M., and Arteta, J. L., in press.

11. Michael, M., Whorton, C. M., Proc. Soc. Exp. Biol. and Med., 1951, v76, 754.

12. Selye, H., *ibid.,* 1953, v82, 328.

13. Menkin, V., *Newer Concepts of Inflammation.* C. Thomas Publishers, Springfield, Ill., U.S.A., 1950.

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Failure of Cortisone and Hydrocortisone to Replace Pteroylglutamic Acid and Citrovorum Factor in Microbial Growth. (20820)

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An unsuspected mechanism by which steroid hormones might influence unicellular metabolism was suggested in the reports of Gaines and her associates indicating that dehydroisoandrosterone(1) and cortisone(2) replace pteroylglutamic acid (PGA) and citrovorum factor (CF) in supporting the growth of certain lactic acid-producing bacteria. These reports conceivably offered a method of investigating steroid hormone action and, in the light of the observed effects of adrenocortical hormones in pernicious anemia(3) and in sprue(4), were of particular interest with respect to hematopoiesis. As a collateral aspect of studies on PGA metabolism in this laboratory, an attempt was made to confirm and to explore further by microbial technics the relationship between cortisone and PGA and CF.

Methods. The methods described by Gaines *et al.*(2) were duplicated as far as possible. The PGA replacing activity of cortisone and of hydrocortisone was assayed with *S. faecalis* 9790, employing the basal medium of Rabinowitz and Snell(5), and with *L. casei* ATCC 7469, employing the medium of Flynn (6). *Le. citrovorum* ATCC 8081, used in the assay of CF activity, was grown in the medium of Sauberlich and Baumann(7), substituting acid-hydrolyzed casein for part of the amino acid mixture. All organisms were grown at 37°C, and growth was measured titrimetrically in all instances after 72 hours' incubation, and turbidimetrically in some instances after 20 hours' incubation. Crystalline cortisone acetate, cortisone, and hydrocortisone[†] were each tested in duplicate at least once with each organism. Alcoholic solutions of steroid were evaporated under vacuum in the assay tubes, pre-autoclaved growth media were added, and the tubes were then shaken for 30 minutes prior to inoculation. Serial concentrations of steroid from 0.1 μg/ml to 500 μg/ml were tested, although undissolved crystals of steroid remained visible in the media at concentrations of 50 μg/ml and above.

Results. No growth was observed at any concentration of cortisone acetate, cortisone, or hydrocortisone with any of the organisms tested. Furthermore, cortisone acetate at a concentration of 50 μg/ml did not potentiate the growth response of *S. faecalis, L. casei,* or *Le. citrovorum* to a wide range of concentrations of PGA and CF.

Summary. Cortisone and hydrocortisone, added to standard assay media, failed to support or to potentiate the growth of *S. faecalis, L. casei,* or *Le. citrovorum.*

1. Gaines, D. S., and Totter, J. R., Proc. Soc. Exp. Biol. and Med., 1950, v74, 558.

2. Gaines, D. S., Broquist, H. P., and Williams, W. L., *ibid.*, 1951, v77, 247.

3. Lowenstein, L., Shapiro, L., and Browne, J. S. L., *Proc. 2nd Clin. ACTH Conference,* The Blakiston Co., 1951, vI, 426.

4. Adlersberg, D., Colcher, H., and Drachman, S. R., *Gastroenterology,* 1951, v19, 674.

5. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.,* 1947, v169, 631.

6. Flynn, L. M., Williams, V. B., O'Dell, B. L., and Hogan, A. G., *Anal. Chem.,* 1951, v23, 180.

7. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.,* 1948, v176, 165.

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[*] Public Health Research Fellow of the National Institute of Arthritis and Metabolic Diseases.

[†] All steroids were kindly supplied by Merck & Co., Rahway, N. J.

Poliomyelitis. IV. Some Cultural and other Characteristics of Chick-Embryo-Adapted Type II Strain of Poliomyelitis Virus. (20821)

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The adaptation of the MEF1, Type II (Lansing) strain of poliomyelitis virus to the developing chick embryo has been reported recently by Roca-Garcia and his associates (1), and by Cabasso and coworkers(2). This adaptation followed the serial propagation of the virus in suckling hamsters by Moyer and coworkers(3). It seems to have been made possible by the continuous passage of the virus in this host, which resulted in a remarkable increase of virus mouse titers. Proof of the identity of the chick-embryo-adapted virus as Type II poliomyelitis was derived from cross-neutralization tests in mice with known standard-type immune sera. Furthermore, evidence of reduced virulence for primates was shown with both hamster and chick-embryo-adapted viruses by intracerebral inoculation into rhesus monkeys(1-3).

This communication presents the results of studies undertaken to determine the optimal conditions for development of the chick-embryo-adapted virus in this foreign host. Particular reference is given to the temperature and length of incubation, route of inoculation, age of embryo and invasiveness of the virus for the various embryonic tissues.

Materials and methods. Virus strains. The strains of virus used were identified previously as MEF1-A and MEF1-B(2). The passage levels of both lines are given in the descriptions of the individual experiments. *Chick embryo inoculation.* Seven-day-old White Leghorn chick embryos were used unless otherwise specified. Inoculation was usually made via the allantoic cavity, although in some experiments, the yolk sac, amniotic sac, and chorioallantoic membrane (CAM) routes were also used. The inoculum consisted of a 20% chick embryo tissue suspension in hormone broth and the following amounts were routinely injected: 0.25 ml in the allantoic cavity, 0.4 ml in the yolk sac, 0.2 ml on the CAM, and 0.1 ml in the amniotic cavity. In most experiments, the whole embryo was harvested and made into a 20% suspension in hormone broth prior to titration in mice. However, in some cases the embryo was decapitated, heads and bodies being separately titrated in mice; in others, the CAM, allantoic fluids and other embryonic parts were also harvested separately for titration in mice. *Mouse titrations.* Three-week-old Swiss albino mice were used for the titration of infected chick embryo fluids or tissues. The latter were extracted in the undiluted form by means of Ten Broeck grinders and the extract was centrifuged at 2,000 rpm for 20 minutes. Serial 10-fold dilutions were then made from the undiluted, centrifuged extract and 4 to 6 mice were inoculated intracerebrally with 0.03 ml volumes of each dilution. Inoculated mice were observed for paralysis and death for a period of 21 days. Virus LD_{50} titers were calculated by the Reed and Muench method(4).

Experimental. Effect of length of incubation on multiplication of chick-embryo-adapted virus. Two experiments were carried out with the MEF1-A strain of virus, using the 3rd and the 24th chick embryo passage levels. Since the results were practically identical only the experiment made with the 24th passage virus is described. Fifty-six, 7-day-old embryos were inoculated with the virus via the allantoic cavity. After sealing with collodion, the embryos were incubated at 36°-37°C. On each of the 9 days, beginning 2 days after inoculation, 6 live embryos were harvested; whole embryos and allantoic fluids were pooled separately. The undiluted embryo pool extract and the allantoic fluid pool from the daily harvests were each titrated in mice by the method described above. The results, Table I, seem to indicate that a post-injection incubation period of 4 to 7 days gives optimal virus titers in the whole embryo extract with maximal titers at 4 and 5 days.

TABLE I. Effect of Length of Incubation on Multiplication of MEF1 Chick-Embryo-Adapted Virus.

	Mouse LD_{50} titers			
	MEF1-A24		MEF1-B24	
Post-inj. incubation, days	Whole embryo	Allantoic fluid	Whole embryo	Allantoic fluid
2	$10^{-3.0}$	$10^{-1.0}$	$10^{-4.41}$	$10^{-4.0}$
3	$10^{-3.0}$	$10^{-2.21}$	$10^{-4.27}$	$10^{-3.74}$
4	$10^{-4.76}$	$10^{-2.33}$	$10^{-5.50}$	$10^{-1.17}$
5	$10^{-5.0}$	$10^{-1.50}$	$10^{-6.50}$	$10^{-1.20}$
6	$10^{-4.33}$	*	$10^{-5.0}$	$10^{-1.20}$
7	$10^{-4.0}$	$10^{-0.5}$	$10^{-4.76}$	$10^{-1.0}$
8	$10^{-3.63}$	$10^{-1.0}$	$10^{-5.30}$	$10^{-1.0}$
9	$10^{-2.33}$	$10^{-1.33}$	$10^{-2.50}$	*
10	$10^{-2.25}$	$10^{-0.}$	†	†

* Not detectable.
† Not done.

In contrast, there was little variation in the allantoic fluid titers, which remained rather low. These low titers may well be due to virus residue from the inoculum rather than to active multiplication of the virus in the surrounding CAM with subsequent virus accumulation in the fluid. A similar test was carried out with the 24th passage level of MEF1-B virus. Its results also summarized in Table I, are strikingly similar to those obtained with the MEF1-A 24 virus, with somewhat higher LD_{50} titers.

Effect of incubation temperature on multiplication of the virus. Duplicate experiments were made with the 26th and the 28th chick embryo passage levels of the MEF1-A virus. The results were so similar that only the latter is described. Forty 7-day-old embryos were each inoculated via the allantoic cavity with the virus suspension. After sealing, they were divided into 4 groups of 10 embryos each, and each group was placed in a separate incubator at 32°C, 35°C, 37°C or 39°C for 5 days. Whole embryos were then harvested from each group separately, ground as described above and titrated in mice. The results, Fig. 1A, seem to indicate that optimal virus titers can be obtained at 5 days by holding the chick embryos at either 35°C or 37°C.

Effect of route of inoculation on multiplication of the virus. Following is the description of one of the experiments comparing the virus titers obtained by different routes of inoculation. Fifty-six 7-day-old embryos were divided into 4 groups of 14 embryos each. They were inoculated with the 28th MEF1-A chick embryo passage virus, the first group in the allantoic cavity (a), the second in the amniotic sac (b), the third in the yolk sac (c), and the fourth on the CAM (d). After sealing, the embryos were incubated at 36°C-37°C, for 5 days. Whole embryos were harvested from each group separately. In addition, CAM's were harvested from groups *a* and *d*. Suspensions of the 6 pools of tissues were titrated in mice, Fig. 1B. It seems that the ability of the virus to invade the embryo proper and to multiply in its tissues is equally good regardless of the route of inoculation. Slightly higher titers were consistently obtained, however, when the allantoic cavity and the yolk sac routes were used. In contrast, multiplication of the virus in the CAM seemed to be of low grade or even non-existent.

Effect of age of the embryo on multiplication of the virus. Groups of 10 to 20 embryos each, varying in age from 5 to 12 days, were all inoculated on the same day, via the allantoic cavity, with the 17th MEF1-A chick embryo passage virus. They were incubated at 36°C-37°C for 5 days, then whole embryos from each group were harvested separately. Embryonic suspensions were titrated in mice in serial, 10-fold dilutions, Fig. 1C. Optimal virus titers were obtained with 6- to 9-day-old embryos. A sudden and drastic change in susceptibility to the virus seems to take place in embryos 10 days of age or older, for their virus titers were very low or insignificant.

Virus distribution in the infected embryo and the effect of inoculum dilution. At frequent intervals pools consisting of infected whole embryos or of separate heads and bodies were titrated in mice. This was done to determine whether the virus multiplied primarily in nervous tissue and whether multiplication also occurred in other embryonic tissues. Effect of inoculum dilution on ultimate virus titers was also investigated. A typical experiment is described as follows: A group of 20 7-day-old embryos was inoculated with the 28th MEF1-A chick embryo passage virus via the allantoic cavity. After 5 days incubation whole embryos were harvested from 10 of the eggs and pooled. The other 10 embryos were

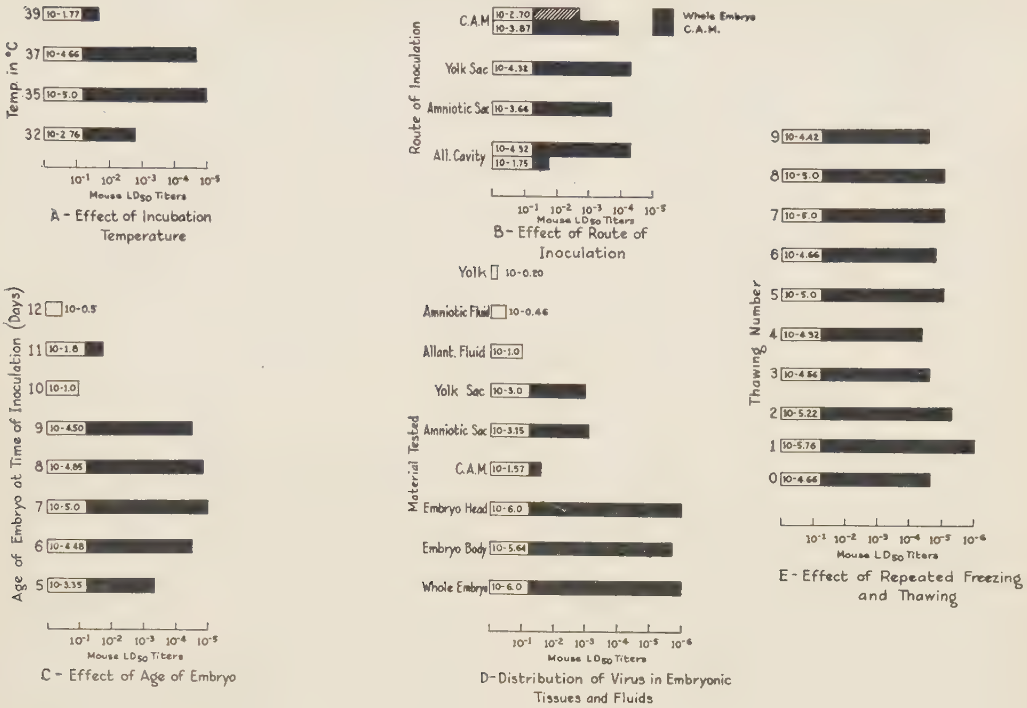

FIG. 1.
Some cultural and other characteristics of a Type II, chick embryo adapted poliomyelitis virus.

decapitated and their bodies and heads pooled separately. Suspensions from each of the 3 pools were in turn inoculated into the allantoic cavity of 20 7-day-old embryos. In addition, serial 10-fold dilutions ranging from 10^{-1} to 10^{-4} were made with the suspension of embryo heads and each dilution was inoculated into the allantoic cavity of 10 7-day-old embryos. Tissues harvested from all groups of embryos after 5 days incubation were titrated in mice. The results appear in Table II. It seems evident that the virus is uniformly distributed throughout the embryo with no particularly greater concentration in the head. In addition, there does not seem to be an optimal inoculum dilution: the greater the concentration of the virus in the inoculum, the greater the virus titer obtained. In addition to determining the virus distribution in the embryo proper, experiments were also devised to study its distribution in other embryonic tissues and fluids. In one such experiment, 20 7-day-old embryos were inoculated via the allantoic cavity with the 41st MEF1-B chick embryo passage virus, which had a mouse LD_{50} titer of $10^{-5.75}$. After 5 days' incubation the following were harvested from 10 chick embryos and the homologous materials pooled: whole embryos, CAM's, amniotic sacks, yolk sacs, allantoic fluid, amniotic fluid and yolk fluid. Tissues were pooled in their entirety whereas only aliquot parts of the fluids were combined. Heads and bodies of the remaining 10 embryos were harvested separately and pooled. Tissue pools were made into 20% suspensions and the 9 tissue and fluid preparations thus obtained were titrated in mice in 10-fold dilutions. The results, Fig. 1D, again seem to indicate that the virus is uniformly distributed throughout the whole embryo. Both allantoic sac and fluid once again were found to be relatively poor in virus content, whereas amniotic and yolk sacs had appreciable titers. Only negligible amounts of virus were found in amniotic and yolk fluids.

Effect of repeated freezing and thawing on infectivity of the virus for mice. It is well

TABLE II. Virus Distribution in Infected Embryos and Effect of Inoculum Dilution.

Chick embryo passage level	Source of inoculum	Dilution of inoculum (20% suspension)	Mouse LD_{50} titers		
			Whole embryos	Embryo bodies	Embryo heads
29	Whole embryos 28th passage	Undiluted	$10^{-4.37}$	$10^{-5.0}$	$10^{-4.75}$
30	*Idem* 29th passage	”	$10^{-4.5}$		
	Embryo bodies 29th passage	”	$10^{-4.0}$	$10^{-3.25}$	$10^{-4.30}$
	Embryo heads 29th passage	”	$10^{-4.48}$	$10^{-4.0}$	$10^{-4.0}$
		1:10	$10^{-4.76}$		
		1:100	$10^{-4.62}$		
		1:1000	$10^{-2.55}$		
		1:10000	$10^{-2.25}$		

known that monkey or rodent-grown poliomyelitis virus can survive in the frozen state for long periods of time and may even remain viable for months when stored at +4°C. The virus also survives repeated freezing and thawing. The following experiment was performed to determine whether this characteristic of poliomyelitis virus was maintained after its adaptation and serial propagation in the chick embryo. A group of 20 7-day-old embryos was inoculated via the allantoic cavity with the 27th MEF1-A chick embryo passage virus. After 5 days' incubation the whole embryos were harvested, pooled, and an undiluted extract prepared as described under *Methods*. A portion of this extract was at once titrated in mice in 10-fold dilutions and 10.0 ml of the remainder were shell-frozen in a glass vial and stored at –35°C. Beginning the next day and every 4th day thereafter, the frozen suspension was thawed under cold running water, and a quantity of material sufficient for one virus titration was removed. The remainder was refrozen and stored. This operation was repeated 9 times. Fig. 1E shows the mouse LD_{50} titers obtained at the different thawings, and indicates that the viability of the chick embryo propagated virus exhibited surprising resistance to 9 consecutive freezings and thawings. It is worth noting that, following the first thawing, the virus titer was found to be over a full 10-fold higher than that of the fresh preparation, an observation which has been made on a great number of other preparations of this virus. It cannot be argued that freezing helps to free more virus particles from cells which remained intact, since the undiluted chick embryo extract was centrifuged prior to freezing. A more appropriate explanation may be that freezing helps disperse virus aggregates present in the fresh extract.

Effect of chick embryo propagated MEF1 virus on hatchability of infected embryos. It was repeatedly observed during the course of this study that although embryos infected with either one of the 2 strains of chick embryo adapted MEF1 virus seemed to overcome the infection around the 12th to 13th day following inoculation, very few chicks hatched out and the great majority died between the 22nd and 26th day of total incubation. The following experiment illustrates the influence of the virus on the hatchability of infected embryos: A total of 60 7-day-old embryos was divided into 3 groups of 20 embryos each and inoculated via the allantoic cavity. Group 1 was inoculated with the 65th MEF1-A passage virus, having a mouse LD_{50} titer of $10^{-5.15}$. Group 2 received the 47th MEF1-B passage virus with an LD_{50} titer of $10^{-5.34}$, and Group 3 was injected with an equal volume of sterile broth diluent. All embryos were incubated at 36°-37°C until they hatched out or died. The results, Table III, show a significant difference between the number of control and infected embryos that hatched out. It may also be observed that strain B seemed to allow fewer embryos to hatch out than did strain A. However, the difference between these may well be within the range of experimental error. It should be

TABLE III. Effect of Chick Embryo Propagated MEF1 Virus on Hatchability of Injected Embryos.

Age of embryo	Virus injected						Broth control		
	MEF1-A65			MEF1-B47					
	Alive	Dead	Hatched	Alive	Dead	Hatched	Alive	Dead	Hatched
8	20			20			20		
10	20			19	1		19	1	
11	20			19			19		
12	20			17	2		19		
13	20			17			19		
14	20			16	1		18	1	
15	20			16			18		
17	19	1		16			18		
18	18	1		15	1		18		
19	18			15			17	1	
20	18			15			17		
21	17	1		15			16	1	
22	15	2		12	3		16		
24	8	1	6	3	6	3	8	2	6
25	5	3		0	3		2	1	5
26	1	4					2		
27	0		1				0		2
Total	0	13	7	0	17	3	0	7	13
%	0	65	35	0	85	15	0	35	65

noted that the temperature of incubation (36°-37°C), though optimal for multiplication of the virus, was substantially lower than the optimal hatching temperature (38°-40°C). This may explain the fact that only 13 out of the 20 control embryos hatched out, with a delay of from 3 to 7 days over the normal hatching time of 21 days.

Summary. 1. Effects of incubation time and temperature, age of embryo and route of inoculation on multiplication of a Type 2 chick-embryo propagated poliomyelitis virus were studied. The distribution of the virus in chick embryo tissues and fluids, and its action on the hatchability of infected embryos, were investigated. 2. Optimal conditions for multiplication of the virus were inoculating 6- to 9-day-old embryos via either the allantoic cavity or the yolk sac, and incubating for 4 to 6 days at 35°-37°C. 3. The best source of the virus was the embryo proper. No greater concentration of virus was found in the head than in the body. 4. Although the embryos seemed to overcome the infection by the 12th or 13th day, relatively few hatched out. The majority died between the 22nd and 27th day of total incubation. 5. The mouse LD_{50} titers of the chick-embryo-adapted virus were essentially unaffected by 9 consecutive freezings and thawings of the same virus preparation.

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1. Roca-Garcia, M., Moyer, A. W., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 519.

2. Cabasso, V. J., Stebbins, M. R., Dutcher, R. M., Moyer, A. W., and Cox, H. R., *ibid.*, 1952, v81, 525.

3. Moyer, A. W., Accorti, C., and Cox, H. R., *ibid.*, 1952, v81, 513.

4. Reed, L. J., and Muench, H. A., *Am. J. Hyg.*, 1938, v27, 493.

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Scintillation Counter Assay of I^{131} and P^{32} in Blood Volume Studies. (20822)

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(Introduced by B. J. Jandorf.)

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The studies of this report were designed to investigate the use of the sodium iodide crystal scintillation counter to determine simultaneously the volumes of distribution of red cells labeled with P^{32} and albumin labeled with I^{131}. Berson and Yalow(1) have used this combination previously measuring the 2 isotopes with separate counting systems. The procedures used in measuring the circulating red cell mass and the plasma volume simultaneously have been reviewed by Berson and Yalow(1) and by Sjostrand(2). In most of these methods chemical as well as isotope labeling is required and a single isotope is used. In the procedure described in this report, 2 isotopes are injected together and the individual activities analyzed from the same sample of whole blood.

Method. Although the well-type sodium iodide crystal scintillation counter was designed primarily for the assay of "gamma" activity from liquid samples, it is also sensitive to high energy beta emissions that penetrate the light shield covering the crystal. The presently used crystal (Harshaw Chemical Co., Cleveland, Ohio) is sheathed with 0.032 inch of aluminum. From P^{32}, a pure beta emitter (beta max. 1.7 mev), 2×10^4 counts per minute per microcurie were obtained. The above mentioned amount of aluminum will absorb approximately 70% of the incident beta rays from the radioactive phosphorus. To avoid the further absorption that would be present with the glass wall of the conventional test tube, a thin walled plastic ("Lusteroid," International Equipment Co., Boston, Mass.) test tube (13 mm I.D.) was used to contain the sample during the counting.

Approximately 5.3×10^5 counts per minute per microcurie were obtained from I^{131} with the equipment used. Separation of the activities of the 2 isotopes was obtained by counting twice; once when the test tube was placed within a brass cup (wall 1 mm thick, equivalent to 840 mg/cm^2) and once without the cup. This thickness of brass is sufficient to prevent penetration of the primary P^{32} beta rays, but counts are still obtained through the brass, presumably as a result of secondary Bremsstrahlung X-rays. With the instrumentation used in the studies of this report, the P^{32} counting rate with the cup was reduced to 20% of that for the same sample without the cup, and the I^{131} counting rate with the cup was 95% of that without it. Changes of these "cup factors" were observed when different photomultiplier tubes were used, but no changes were found with a given tube. Factors (f_P and f_I) varying between 15 and 30% were found for the P^{32} counting rates and between 91 and 98% for the I^{131}. Individual activities of the 2 isotopes were calculated by solving the following simultaneous equations:

$$\begin{aligned} \text{Counts without cup} &= I^{131}\text{ counts} + P^{32}\text{ counts} \\ \text{Counts with cup} &= f_I\, I^{131}\text{ counts} + f_P\, P^{32}\text{ counts} \end{aligned}$$

It was found necessary to have high counting rates and to adjust the relative activities so that 2 to 3 times as many counts were obtained from the phosphorus as from the

TABLE I. *In Vitro* Studies.

Diluent	I^{131} (iodinated albumin)			P^{32} (sodium phosphate)		
	Alkaline saline		Blood	Alkaline saline		Blood
	Unmixed*	Mixed*		Unmixed*	Mixed*	
Avg of 10 samples, counts/min.	2993	2927	2963	7927	7793	7470
Coefficient variation	1.9%	2.8%	2.6%	1.0%	1.9%	2.6%
Ratio: Mixed/unmixed		.98	.99		.98	.94

* See text for explanation of unmixed and mixed solutions.

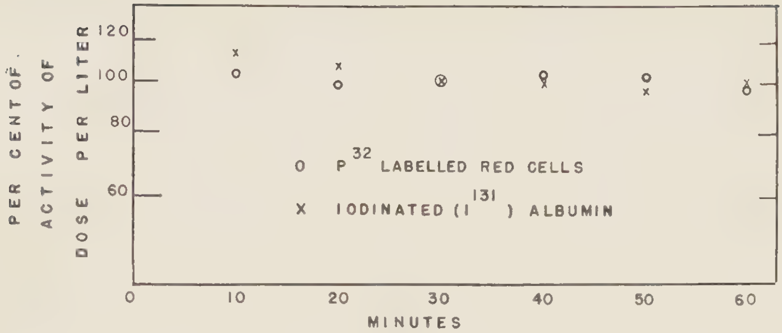

FIG. 1.

iodine. In the present experiments, the average counts were approximately 6,000 counts per minute for the P^{32} and 2,500 counts per minute for the I^{131}. Three-minute counting periods were used and each sample was counted in duplicate. The iodinated (I^{131}) human serum albumin and a sterile buffered sodium phosphate (P^{32}) solution were obtained from Abbott Laboratories, North Chicago, Ill.

In Table I are presented data obtained from an *in vitro* "recovery" study. From a solution containing P^{32} (sodium phosphate) and from a separate one containing I^{131} (iodinated albumin), 1/20 dilutions were made and counted separately (unmixed values, Table I). Equal parts of the 2 solutions were then mixed and recounted at a 1/10 dilution, diluents being saline containing 0.2% NaOH and whole heparinized blood. These samples were counted both with and without the cup and the separate activities of the 2 isotopes were calculated (mixed values, Table I). The observed lower counts from whole blood, as compared to saline, are considered due to the difference in the density of the 2 solutions; blood 1.057 g/cm³, alkaline saline 1.012 g/cm³. Two specific problems were encountered in the handling of these radioactive agents. First, with high dilutions of the iodinated albumin it was noticed that as much as 20% of the activity appeared to be "lost". Attraction of the protein to the wall of the glassware was considered responsible and the effect was not observed if amounts of carrier albumin, in the form of a few ml of plasma, were added to the primary dilution. This precaution was suggested by Dr. C. Leewallen of the Brookhaven National Laboratory. Secondly, it was observed that the P^{32} activity in the counting tubes appeared to increase, when counted on successive days, if neutral water or saline solutions were used. This effect was explained by postulating adherence to the wall of the plastic tube, thus changing the geometry so that less of the beta energy was absorbed in the water and a higher counting efficiency resulted. The phenomenon was not observed when the diluent was whole blood or alkalinized saline.

In vivo dilution measurements. The results of a preliminary experiment describing the blood dilution values of P^{32} tagged red cells and of iodinated (I^{131}) human serum albumin in a 10 kg dog are plotted in Fig. 1. The dog red cells were labeled in the manner described by Hahn and Hevesy(3) and as modified by Berson and Yalow(1), but a higher dosage of P^{32} was used than was recommended by either of these authors. The final cell preparation contained approximately 100 microcuries of P^{32}. A few ml of plasma containing 1.5 microcuries of iodinated (I^{131}) albumin were then added to the cell suspension and mixed thoroughly. Prior to the injection into the dog, an aliquot of the mixture was taken and diluted in alkaline saline to serve as a reference standard from which to analyze the dose per liter activity concentration for each agent. Blood samples were drawn at 10-minute intervals. Three ml amounts of heparinized whole blood were counted and the separate activity concentration for each isotope was determined as described. The P^{32} counts from blood were divided by the factor of 0.96 to correct for the loss of counts from the somewhat denser blood as compared to the alkaline saline standard. Red cells were prevented from settling in the whole blood samples by manual agitation with wire loops prior to the counting. The cells were hemolyzed with saponin in the standard solution. The results (Fig. 1) show close superimposition of the values indicating near equality of the volumes of distribution of these 2 agents.

Summary. The use of the well-type sodium iodide crystal scintillation counter to measure P^{32} and I^{131} from a single sample of whole blood is described. The individual activities are calculated from data obtained with and without a metal cup absorber.

1. Berson, S. A., and Yalow, R. S., *J. Clin. Invest.*, 1952, v31, 572.
2. Sjostrand, T., *Physiol. Rev.*, 1953, v33, 202.
3. Hahn, L., and Hevesy, G., *Acta Physiol. Scandinav.*, 1940, v1, 3.

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Phosphate (P^{32}) Exchange in Brain Phospholipids *in vivo* and *in vitro*.*†

(20823)

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It is now well established that adult neural tissue can synthesize phospholipids *in vitro* as well as *in vivo*(1-7), and the inference that these "structural" components of the cell are involved in normal functional metabolism has some direct support(6-8). On the other hand, the slow phosphorus turnover of brain phospholipids would preclude a very active metabolism unless the apparent low overall specific activity in fact represents high activity of a small fraction. In favor of this, it had been shown(1,2) that P^{32} is incorporated to a greater extent into brain cephalins and sphingomyelin than into lecithins; and in *in vitro* studies we observed much higher specific activities in some lipid fractions than in others. Further study shows a highly active protein moiety (derived from lipoprotein) which may represent a part of the inositol metadiphosphate complex isolated by Folch and Lees(9).

Methods. *In vitro,* the brains of 6 to 12 male Sprague-Dawley rats were used in each experiment. Each brain was homogenized in 8.0 cc of 0.01 M potassium phosphate buffer (pH 7.4) immediately after it was removed from the skull and kept at –7°C until the brain tissues of the different animals were combined. The entire homogenate, together with $Na_2HP^{32}O_4$, glucose (10^{-1} M), K ATP (3.1×10^{-4} M), DPN (6.1×10^{-5} M), cytochrome c (5×10^{-5} M), $MgCl_2$ (8.0×10^{-3} M), and KF (8.0×10^{-3} M) was incubated at 37°C in the Warburg water bath. After shaking for 20 minutes, one hour, or 2 hours, the tissue was separated from the incubating medium by centrifugation at –7°C for 20 minutes at an R.C.F. of 2700 times gravity. The lipids were extracted from the brain tissue at 4°C with 20 volumes of cold chloroform-methanol (2:1) per g of tissue(9), and the extract was separated from the residue by filtration at 4°C. The residue was reextracted 2 more times, the 3 filtrates were combined, and were washed several times with distilled water at 4°C by the method of Folch(9) to remove water-soluble contaminants. The chloroform-methanol extract was then dried *in vacuo*. This process released some of the protein from lipoprotein elements which were soluble in chloroform-methanol(9).

The lipids were redissolved in 20 volumes

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† Supported by contract between the Office of Naval Research and the University of Chicago.

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TABLE I. Specific Activities of Various Fractions after Incubation of Brain Homogenates with P^{32} at 37°C.

Time of incubation	Inorganic phosphate*	Delta 7*	Organic acid-soluble*	Lipid extract	Lipid / Delta 7
20 min.	1188	929	753	1.1	1.2×10^{-3}
1 hr	1132	972		1.1	1.1 "
2 "	1124	945	737	1.2	1.3 "

* Brain tissue was separated from medium by centrifugation. These fractions were isolated from supernatant.

TABLE II. Specific Activities of Various Crude Fractions Obtained from Lipid Extracts of Brain Tissue.

Fraction	% total PL-P	*In vitro*. Separation of protein:			*In vivo*
		Optimal	Partial	Omitted	
Inorganic phosphate of medium		1025			
Entire lipid extract*	100	1.0	1.0	1.0	1.0
Protein	1–2	20.4	13.0		.77
Acetone soluble	1–2	.25	.84		
Methanol "	88–95	.20	.62	.55	.92
Ether "	2–6	5.32	6.19	9.48	.73
Chloroform-methanol soluble	0.5–3	2.71	8.07	17.4	.99

* Specific activity of entire lipid extract designated as unity.

of chloroform-methanol and separated from the insoluble protein components by filtration at 4°C. The protein moieties were washed 6 to 10 times with a total of over 100 volumes of chloroform-methanol to remove adhering lipids. The lipids which had been redissolved were dried *in vacuo* again. The dry residue was then extracted at 0°C 5 times with a total of 100 volumes of each of the following solvents: acetone, followed by 95% ethyl alcohol, and finally diethyl ether. The residue was redissolved in chloroform-methanol. Although each of these fractions contains a mixture of substances(10), the choline lipids are found predominantly in the methanol extract, whereas the cephalins are soluble, for the most part, in ether and chloroform. The specific activities (counts/min./μg of phosphorus) of the entire extract and of the different fractions were measured after ashing in 10 N H_2SO_4 for 12 hours at 110°C. The exchange of P^{32} in the acid-soluble lipoproteins was not investigated. In one experiment, the specific activity of the phosphate of the incubating medium was measured. Inorganic and delta 7 phosphates were precipitated as $MgNH_4PO_4$, and the remaining "organic acid-soluble" phosphate (representing mainly the phosphate of the glycolytic intermediates) was obtained by ashing at 110°C in 10 N H_2SO_4 for 12 hours. The specific activities of these components were ascertained after incubation times of 20 minutes, one hour, and 2 hours. Also, in one experiment, sphingomyelin was isolated according to the method of Brante(10).

In vivo, 375 μc of P^{32} were injected intraperitoneally into each of 4 rats, and the animals were sacrificed 20 hours later. The lipids obtained from the brains of these animals by homogenization in chloroform-methanol were washed and divided into crude fractions as described above. In other experiments, rats were injected with 200 μc of P^{32} and sacrificed 20 minutes, one hour, 4 hours, and 20 hours later. In this case, the lipids were isolated in a single fraction by the method of Schneider (11) and the specific activity of the entire extract was measured.

Results. In vitro, the total phospholipids as well as the acid-soluble components of the medium reached their maximum specific activities within 20 minutes after the start of incubation and then held constant through the 2-hour period (Table I). The lipid extract attained a specific activity approximately 1/1000 that of the inorganic phosphate of the medium.

TABLE III. Specific Activities of Lipid and Delta 7 Fractions of Normal Rat Brain at Various Times after Intraperitoneal Injection of 200 Microcuries of P^{32} per Animal.

Time	No. animals	Lipid (±1 S.D.)	Delta 7 (±1 S.D.)	$\frac{\text{Lipid}}{\text{Delta 7}}$
20 min.	3	.057 (±.018)	21.3 (± 4.3)	2.6×10^{-3}
1 hr	3	.24 (±.016)	39.3 (± 7.1)	6.1×10^{-3}
4 "	3	.95 (±.26)	34.6 (± 4.0)	2.7×10^{-2}
20 "	2	3.0 (±.0)	29.8 (± 2.6)	1.0×10^{-1}

In other experiments the lipids were divided into crude fractions as described above. Taking the specific activity of the entire phospholipid extract as unity, values of the fractions varied from 20.4 for the protein to 0.2 for the methanol portion—a 100-fold range (Table II). Sphingomyelin, actually isolated from the lipid fraction, had a specific activity of only 0.09 on this scale; and, conversely, when the preliminary separation of the protein was less complete, or was omitted, its activity appeared mostly in the chloroform-methanol fraction (Table II).

In vivo, by contrast, the specific activity of the total phospholipids rose continuously for the 20-hour period (roughly linearly during the first few hours after the intraperitoneal administration of P^{32} (Table III), and a considerably greater amount of P^{32} was eventually incorporated *in vivo* than *in vitro* (see also(4)); approximately 80 times as much after 20 hours, if it is assumed that the delta 7 fraction comprises the source of the phospholipid phosphate. Moreover, all fractions showed approximately the same specific activity, the range being 0.73 to 0.99 (Table II).

Discussion. Two points require attention in comparing *in vivo* and *in vitro* results: 1) the much reduced turnover *in vitro,* both in time to equilibrium and in total; and 2) the great quantitative differences in turnover between fractions *in vitro* but not *in vivo.* A loss of general synthetic power *in vitro* is understandable as a result of the limited availability of adenosine triphosphate(14), dilution of reactants, enzyme destruction(3), or activation of autolytic reactions(12) consequent to the disruption of structure. For example, the relative excess of breakdown to synthesis in brain homogenates decreases the total lipid content by 10% to 15% after several hours incubation(4,13). The second point, that *in vitro* turnover is perhaps limited to one or 2 "trace" components of the total phospholipids, can be interpreted along similar lines if it be assumed that the active substances normally serve as phosphate carriers to the other phospholipids. *In vivo* all steps in the sequence are intact and the specific activities rise more or less together; *in vitro* the lipoprotein step primarily remains, with the results observed.

In *in vitro* experiments it was observed that the "active" material of the protein moiety was predominantly chloroform soluble. Since the protein fraction actually contains diphosphoinositide(15) which is soluble almost exclusively in wet chloroform, it is possible that this substance contains most of the P^{32} incorporated into the protein fraction. However, the relatively high specific activity of the ether soluble material indicates that other cephalins are also involved in phosphate exchange *in vitro.*

Summary. In vitro, the specific activity of the phospholipid phosphate reaches a maximum within 20 minutes after the start of incubation, while *in vivo* the specific activity of this fraction rises continuously for at least 20 hours after the intraperitoneal administration of P^{32}. When the lipid extract is divided into crude fractions, it is observed in *in vitro* experiments that the specific activities of these fractions are markedly dissimilar. Conversely, the specific activities of these fractions are approximately the same when obtained from the brains of rats that had been injected with P^{32} 20 hours previously. Some of the factors involved in these findings have been discussed.

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1. Chargaff, E., Olson, K. B., and Partington, P. F., *J. Biol. Chem.,* 1940, v134, 505.

2. Hevesy, G., and Hahn, L., *Kgl. Denske Videnskab. Selskeb. Biol. Medd.,* 1940, v15, 5.

3. Chaikoff, I. L., *Physiol. Rev.,* 1942, v22, 291.

4. Fries, B. A., Schachner, H., and Chaikoff, I. L., *J. Biol. Chem.,* 1942, v144, 59.

5. Schachner, H., Fries, B. A., and Chaikoff, I. L., *ibid.*, 1942, v146, 95.
6. Dawson, R. M. C., and Richter, D., *Proc. Roy. Soc. B*, 1950, v137, 252.
7. Streicher, E., and Gerard, R. W., in preparation.
8. Gerard, R. W., *Physiol. Rev.*, 1932, v12, 469.
9. Folch, J., and Lees, M., *J. Biol. Chem.*, 1951, v191, 807.
10. Brante, G., *Acta Physiol. Scand.*, 1949, v18, Supplement 63.
11. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.
12. Commoner, B., *Quart. Rev. Biol.*, 1942, v17, 46.
13. Tyrrell, L. W., *Nature*, 1950, v166, 310.
14. Meyerhof, O., *Arch. Biochem.*, 1947, v13, 485.
15. Folch, J., personal communication.

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Ultra-microdetermination of Arginine by a Compound Microbiological Assay Method.* (20824)

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Most microbiological assay methods are based on the principle that an assay organism grows and produces acid proportionately to the concentration of an essential nutrient added at different levels to a basal medium lacking this substance but otherwise nutritionally complete. In amino acid assays the ratios between the moles of acid produced and nutrient added range from about 600:1 (glutamic acid) to 11,000:1 (tryptophan). Magnification in response evidenced by such ratios is a general characteristic of titrimetric microbiological assays.

From unpublished studies in this laboratory it became apparent that the magnification in response inherent in a given microbiological assay could be compounded by means of a 2-stage process in which the growth of a primary organism is regulated by the level of an essential nutrient added to the basal medium and that of a secondary organism by the level of an essential nutrient introduced into the basal medium as a metabolic product of the primary organism.

The determination of an amino acid by a compound microbiological assay procedure is described for the first time in this report. *Lactobacillus casei* 280-16 was selected as the secondary assay organism because of its response to D-lactic acid, a metabolic product of many assay organisms(1). The anticipated gain in sensitivity with this secondary assay was about 2000-fold since the quantity of L-lactic acid produced (available for titration) by *L. casei* 280-16 has been shown to be about 2000 times that of the D-lactic acid available for the growth of this organism(1). *Lactobacillus casei* ϵ (American Type Culture Collection No. 7469) was selected as the primary assay organism because it produces only relatively small amounts of D-lactic acid (1). The resulting relatively low effective magnifications (ratios between D-lactic acid produced and nutrient added) in primary assays with this organism seemed desirable in preliminary studies to avoid difficulties which might be anticipated in compound assays with the highest over-all magnifications. Arginine was selected as the test amino acid since it may be determined satisfactorily in biological materials by a standard (one-stage) assay with *L. casei* ϵ(2).

The compound microbiological assay method described in the present report for the determination of arginine with *L. casei* ϵ and *L. casei* 280-16 in combination has proved to be approximately 140 times as sensitive as the standard procedure while retaining the advantage of conventional macroscale manipulations.† Since other organisms commonly employed in standard assays produce proportionately much more D-lactic acid than

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L. casei ϵ, it is anticipated that compound assay methods may be made available in which the over-all increases in sensitivity are several thousand fold. Studies involving the determination of amino acids in biological materials readily obtainable only in small quantities would be greatly facilitated if microbiological assay procedures of high sensitivity were available. For example, 2000 specimens of *Leptinotarsa decemlineata* were required to furnish blood sufficient for the microbiological determination of 13 amino acids in the deproteinized plasma of this insect(3). One specimen would have sufficed if a compound assay procedure of 2000-fold over-all sensitivity had been available.

Preparation of standard. Aliquots of a stock solution containing 1.000 mg of L-arginine per ml[‡] in 0.5 *N* hydrochloric acid were neutralized approximately to pH 7.0 immediately before use and diluted to give 2 working standard solutions containing, respectively, 10^{-4} and 5 x 10^{-4} mg of L-arginine per ml. From one to five 0.2 ml aliquots of the 2 solutions were pipetted[§] into 13 x 100 mm soft glass test tubes in combinations yielding from 0 to 3 x 10^{-4} mg of L-arginine per tube in steps increasing by 2 x 10^{-5} mg per tube. Triplicate tubes were employed for each of the resulting 15 standard dosage levels. *Preparation of samples.* Protein samples were hydrolysed by refluxing them for 20 hours in 10 volumes of 8 *N* hydrochloric acid. An appropriate aliquot of each hydrolysate, neutralized approximately to pH 7.0 immediately before use, was diluted to contain approximately 2.5 x 10^{-4} mg of L-arginine per ml. One to five 0.2 ml aliquots of each sample solution were pipetted into test tubes by the same procedure as that employed with the standard solutions. Triplicate tubes were used for each of the resulting 5 sample dosage levels. A sufficient number of 0.2 ml portions of water were added to the sample and standard tubes to bring the volume in each to 1.0 ml, and the assay medium was added as described below.

Primary assay medium. Sufficient medium for 100 assay tubes was prepared by dissolving 37.5 mg of amino acid mixture,[‖] 61.8 mg of vitamin-mineral mixture,[¶] 30 mg of calcium acetate monohydrate, and 600 mg of glucose in 60 ml of hot water[**] followed by the addition of 15 ml of buffer solution,[††] 17.5 ml of 3 *N* sodium chloride solution and sufficient water to make 100 ml. The medium was filtered and 1.0 ml was pipetted into each assay tube. The complete rack of assay tubes constituting an entire experiment was closely covered with a sheet of clean aluminum foil and autoclaved for 15 minutes at 15 lb. The tubes were allowed to cool without agi-

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[†] The effective magnification in the primary assay with *L. casei* ϵ was expected to be only about 3% of that found by direct titration since only about 3% of the titratable acid produced by this organism is D-lactic acid(1). On this basis the over-all gain in sensitivity would be 60 fold (3% of 2000, the anticipated gain in sensitivity in the secondary assay). The actual gain was more than twice that predicted due to improvements in experimental conditions.

[‡] The monohydrochloride (C. P. product of Amino Acid Manufactures). The stated concentrations are calculated for the free amino acid.

[§] All repetitive pipettings were made with the aid of a Brewer automatic pipet (Baltimore Biological Co.).

[‖] A dry stock mixture was prepared by grinding the following constituents together in a mortar: 9.28% each of DL-alanine, L-cysteine.HCl, L-glutamic acid, DL-leucine, DL-lysine.HCl, and DL-serine; 4.64% each of L-aspartic acid, glycine, DL-isoleucine, DL-phenylalanine, DL-threonine, DL-tyrosine, and DL-valine; and 2.37% each of L-histidine.HCl.H_2O, DL-methionine, DL-norleucine, L-proline, and DL-tryptophan.

[¶] A dry stock mixture was prepared by grinding the following constituents together in a mortar: $MgSO_4$ (anhydrous) 47.5%, inositol 12.17%, 5.84% each of adenine sulfate, guanine hydrochloride, uracil and xanthine, choline chloride 4.87%, $FeSO_4.6H_2O$ 4.55%, $MnSO_4.H_2O$ 3.70%, 0.97% each of riboflavin and niacinamide, 0.49% each of thiamine.HCl and calcium d-pantothenate, pyridoxine.HCl 0.78%, 0.049% each of pyridoxamine.2HCl, pyridoxal.HCI and *p*-aminobenzoic acid, and 0.0024% each of biotin and folic acid.

[**] A small amount of relatively insoluble material was left undissolved in preference to heating the mixture excessively.

[††] The aqueous solution contained ammonium acetate 11.53%, sodium acetate 11.72%, and 1.0% each of potassium monohydrogen and dihydrogen phosphates.

tation and were immediately inoculated as described below.

Primary inoculation. The stock culture of *L. casei* ϵ (A.T.C.C. 7469) was maintained in Yeast-Dextrose Agar (Difco product) stabs. Serial transfers were made at 3-week intervals and the resulting cultures were stored in a refrigerator after about 40 hours of incubation at 35°C. A liquid inoculum medium was prepared by dissolving 10 g of Bacto-Peptone (Difco product), 5 g of Bacto-Yeast Extract (Difco product), 206 mg of vitamin-mineral mixture,¶ and 20 g of glucose in 500 ml of hot water** followed by the addition of 50 ml of buffer solution†† and sufficient water to make one liter. This medium was covered with a layer of toluene and stored in a refrigerator. A 15-ml aliquot was placed in a 50-ml centrifuge tube. The tube was plugged with gauze-covered cotton, autoclaved 15 minutes at 15 lb, cooled without agitation, inoculated immediately from the stab culture and incubated for 18 hours at 35°C. The resulting growth was washed by centrifuging it (the cotton plug was secured at the top of the centrifuge tube with a rubber band), resuspending it aseptically in 15 ml of sterile 0.85% saline solution, and repeating the process 3 times. One-tenth ml of the final suspension was added aseptically to 100 ml of sterile 5.4% glucose solution, and 1.0 ml of the resulting mixture was pipetted aseptically into each (previously sterilized) assay tube. The aluminum foil cover, which had been removed (and the underside kept sterile) during the few minutes necessary to add the inoculum mixture to the assay tubes, was replaced carefully to avoid bacterial contamination and the rack of assay tubes was incubated in a precision water bath at 35°C for from 65 to 70 hours. At the end of this incubation period the resulting cultures (the amounts of growth obtained were insufficient to be evident by visual observation) were sterilized by steaming for 30 minutes at 100°C in the autoclave, cooled and immediately supplemented with the inoculated secondary medium as described below.

Secondary assay medium. Sufficient medium for 100 assay tubes was prepared in 2 parts as follows: Part I was prepared by dissolving 20.6 mg of vitamin-mineral mixture¶ and 2.4 g of N-Z-Case (an enzymatic digest of casein supplied by Sheffield Farms Co.) in about 40 ml of hot water** followed by the addition of 5 ml of buffer solution†† and sufficient water to make 50 ml. The resulting solution was filtered and autoclaved in a cotton stoppered flask for 15 minutes at 15 lb. Part II was prepared by dissolving 2.0 g of glucose in about 40 ml of hot water followed by the addition of 5.50 ml of *N* hydrochloric acid and sufficient water to make 50 ml. The resulting solution was autoclaved in a cotton stoppered flask for 15 minutes at 15 lb. The 2 parts were combined aseptically after cooling and were inoculated as described below.

Secondary inoculation. The culture of *L. casei* 280-16 described previously(1) was maintained in the manner described above for *L. casei* ϵ, and the inoculum suspension was prepared following the procedure described above for the primary inoculation except that only one washing with sterile saline was employed. Six-tenths of one ml of the final suspension of *L. casei* 280-16 was added aseptically to the 100 ml of sterile secondary medium, 1.0 ml of the resulting mixture was pipetted aseptically into each assay tube employing the same procedure as for the primary inoculation, and the rack of tubes was replaced in a precision 35°C water bath for an additional 40 hours of incubation.

Titration and calculation. At the end of the final incubation period growth was terminated by steaming the rack of tubes in the autoclave for 20 minutes at 100°C. The contents of each triplicate set of tubes were combined and rinsed into a 50-ml Erlenmeyer flask with an equal volume of water. Three drops of an 8 mg per ml solution of bromthymol blue in 50% ethanol were added to each flask and the mixtures were titrated to pH 7.0 (blue-green end point) with standardized approximately 0.1 N NaOH.‡‡ The standard titration values were plotted graph-

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‡‡ This procedure of titrating the combined triplicate cultures was found to give nearly the same results with nearly the same probable errors as that of titrating the separate cultures and averaging the separately determined values.

TABLE I. Percentages of L-Arginine Found in Casein, Gelatin, and Silk Fibroin by the Standard and Compound Microbiological Assay Methods.* Eight trials.†

Casein		Gelatin		Silk fibroin	
Type of microbiological assay					
Standard	Compound	Stand.	Comp.	Stand.	Comp.
%					
3.72	3.26	8.84	8.19	1.04	.99
3.70	3.43	8.68	9.39	1.01	1.04
3.68	3.50	8.70	9.28	1.03	.97
3.68	3.67	9.02	9.93	1.04	1.15
3.62	3.65	8.78	8.92	1.00	1.08
3.65	3.58	8.77	8.75	1.01	.89
3.62	3.25	8.70	9.33	.99	1.10
3.68	3.58	8.76	8.57	1.02	.91
Averages					
3.67	3.49	8.78	9.04	1.02	1.02
Probable error of each trial calculated as % of avg					
.66	3.2	.84	4.1	1.2	6.1
Probable error of avg value calculated as % of avg					
.23	1.1	.30	1.4	.43	2.2
Ratio between probable errors					
1 : 4.8		1 : 4.9		1 : 5.1	

* All arginine values calculated on basis of moisture and ash-free protein. The casein and silk fibroin were the products described previously (4). The gelatin was a first extraction pigskin product of the Knox Gelatin Company. Values for arginine are in good agreement with those reported in the literature. Included among the latter are casein 3.7%, avg of values from 9 laboratories ranging from 3.6 to 3.9% (5); gelatin 9.0%, avg of values from 6 laboratories ranging from 8.0 to 9.3%; and silk fibroin 0.86%, avg of values from 5 laboratories ranging from 0.80 to 0.90% (6).

† Trials 1 through 4 were made simultaneously, employing the same sample and standard solutions, media and inocula. A separate standard curve included in each trial. Trials 5 through 8 were likewise made simultaneously (but not together with trials 1 through 4).

ically against the standard L-arginine dosages per tube. The amounts of L-arginine per tube corresponding to each of the 5 sample dosages in each case were estimated by interpolating the standard curve. The resulting arginine values were converted to g per 100 g of protein, and the values corresponding to the 5 dosage levels were averaged. If one of the 5 values differed from the value nearest it by an amount greater than the entire range of the remaining 4 values it was excluded in calculating the average.

The reliability of the compound microbiological assays was determined by comparing the results by this method with those obtained for the same samples by the standard microbiological assay method described previously (2). The results are given in Table I and Fig. 1 and 2.

Discussion. The experiments leading to the compound microbiological assay method presented above have been too numerous to describe in the limited available space. They have indicated, however, that apparently minor deviations from the described procedure may lead to entirely unsatisfactory results. Excessively high blanks and erratic results were avoided by autoclaving the bulk of the glucose separately from the other constituents of the basal media, employing not more than the indicated quantities of amino acid mixture or of inoculum in the primary medium, utilizing a secondary incubation period of not more than 40 hours, adjusting the acidity of the secondary medium to pH 5.7 optimum for the response of *L. casei* 280-16 to D-lactic acid, and guarding against contamination of reagents and glassware with traces of arginine. It became evident that more rigid control of conditions, more care in manipulations and greater precautions to prevent accidental contamination were required for successful assays by the compound (2-stage) than the standard (one-stage) method.

The component responses made use of in the compound microbiological assay of arginine are shown in Fig. 1 (Curve B) and 2. Curve B (Fig. 1) depicts the response of *L. casei* ϵ to arginine under standard assay conditions. Under somewhat modified conditions this response constituted the first of the 2 consecutive responses obtained in the compound assay. The dosages of arginine ranging up to 1.7×10^{-7} moles in Curve B may be seen to have effected the production of up to 3.1×10^{-4} moles of lactic acid per tube, and hence it is evident that an average magnification of about 1800 ($3.1 \times 10^{-4}/1.7 \times 10^{-7}$) times was attained over this response range. If it is assumed that the characteristics of this response were retained under the modified conditions employed in the compound assay, the average magnification achieved in the primary assay may be calculated to have been about

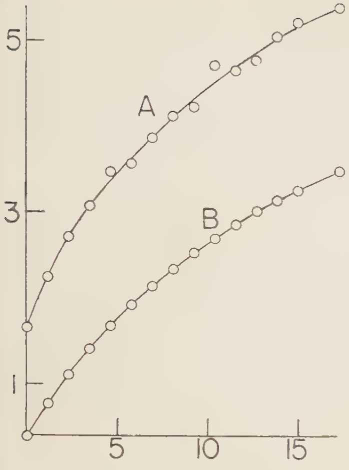

Fig. 1

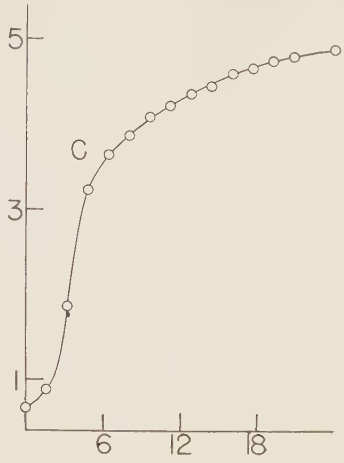

Fig. 2

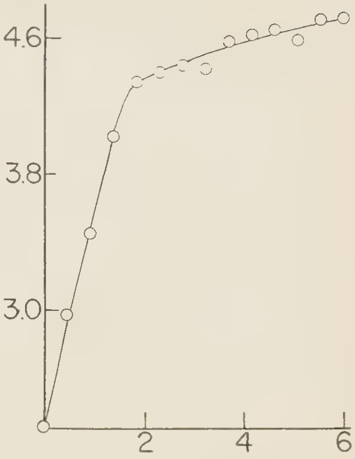

Fig. 3

FIG. 1. Arginine standard curves obtained by the compound (Curve A) and standard (Curve B) microbiological assay methods. The values on the vertical scale are the moles × 10^4 of acid (calculated as lactic acid) produced per tube. The values on the horizontal scale are (Curve A) the moles × 10^{10} and (Curve B) the moles × 10^8 of L-arginine per tube.

FIG. 2. Response of *L. casei* 280-16 to calcium D-lactate under conditions comparable to those employed in the compound microbiological assay of arginine. The values on the vertical scale are the moles × 10^4 of L-lactic acid produced per tube. The values on the horizontal scale are the moles × 10^8 (calculated as D-lactic acid) of calcium D-lactate per tube.

FIG. 3. L-leucine standard curve obtained by a compound microbiological method employing *L. arabinosus* 17-5 as the primary assay organism. The values on the vertical scale are the moles × 10^4 of L-lactic acid produced per tube. The values on the horizontal scale are the moles × 10^{10} of L-leucine per tube. The data were taken from a paper presented by one of the present authors (M.N.C.) at the December 11th meeting of the Southern California Section, Soc. for Exp. Biol. and Med., Los Angeles, 1952.

54 (3% of 1800) times, since only D-lactic acid is effective in promoting the secondary response, and since this isomer constitutes only 3% of the lactic acid produced by *L. casei* ϵ(1). The response of *L. casei* 280-16 to D-lactic acid under the conditions employed in the compound assay is depicted in Fig. 2 (Curve C). Dosages of D-lactic acid up to about 7.4 x 10^{-8} moles may be seen (Curve C) to have effected the production of up to 3.1 x 10^{-4} moles of L-lactic acid per tube. The average magnification over this range in the secondary assay may therefore be calculated to have been about 4200 (3.1 x 10^{-4}/7.4 x 10^{-8}) times.

A typical arginine standard curve obtained by the described compound microbiological assay procedure is shown in Fig. 1 (Curve A). Dosages of arginine up to only about 1.2 x 10^{-9} moles were sufficient, under these conditions, to elicit the final production of up to 3.1 x 10^{-4} moles of L-lactic acid per tube, and hence the average resultant magnification over this range was about 260,000 (3.1 x 10^{-4}/1.2 x 10^{-9}) times. This value is in good agreement with the product (230,000) of the magnifications (54 and 4200) calculated for the 2 component assay stages. Owing to the high magnification of the compound assay, very small samples were sufficient for the determination of arginine by this procedure. The total amounts of sample (sufficient for triplicate determinations at each of 5 dosage levels) employed for each compound assay of arginine were as follows: casein 45 μg, silk fibroin 142 μg, and gelatin 18 μg. One hundred times these amounts were needed for the standard microbiological assays of this amino acid. The percentages of arginine found in casein, gelatin and silk fibroin in 8 consecutive trials by the standard and compound microbiological assay methods are listed in Table I. The probable errors calculated for the individual values were approximately 2 times greater for silk fibroin than for casein by either method, whereas those for gelatin were between these 2 extremes. In spite of these relatively large differences a nearly constant ratio (about 5:1) was observed between the probable errors calculated for the values obtained by the compound method and those calculated for the values found by the standard method. The somewhat lesser precision of the compound method was also indicated by the poorer alignment of points on the standard curve (Curve A, Fig. 1) and by the poorer agreement between the values obtained by this method at different sample dosages (the mean deviations of the values obtained at 4 or 5 different sample dosage levels from their means averaged 7.4% with the compound method as compared with only 1.1% with the standard method for all the values listed in Table I).

The difference (4.9%) between the average values (3.67% and 3.49%, Table I) found for arginine in casein by the 2 assay methods was approximately 3.7 times greater than the sum of their probable errors (0.23% and 1.1%). Since the odds against a chance discrepancy of this magnitude between average values with these probable errors may be calculated to be about 78 to 1, the data appear to indicate a small systematic difference in the relative responses to arginine in casein hydrolysate obtained by the standard and compound microbiological assay methods. On the other hand, the difference (3.0%) between the average values (8.78% and 9.04%, Table I) found for arginine in gelatin by the 2 methods appears to be a chance discrepancy, and the values (1.02% and 1.02%, Table I) found for arginine in silk fibroin by the 2 methods were the same. It is evident that the described compound method would yield erroneously high arginine values for samples containing large amounts of D-lactic acid (the apparent activity of this substance due to its effect in the secondary assay may be calculated to be about 3% of that of arginine), but an error of this sort was not apparent in the above results. D-lactic acid may be removed from acidified sample solutions by extraction with ether, but it seems likely that this step would be required only in special cases.

It should be emphasized that applications of the compound microbiological assay principle do not appear to be limited to the determination of arginine, nor does the sensitivity attainable by the application of this principle appear to be limited to that attained by the method described in the present report. On the contrary, it seems likely that the present procedure could be adapted successfully to the ultra-microdetermination of any one of the amino acids essential for growth and acid production of *L. casei* ϵ merely by adding arginine to the amino acid mixture of the primary medium and omitting, instead, the amino acid to be determined. That primary assay organisms which produce higher percentages of D-lactic acid may lead to assays of greater sensitivity than those employing *L. casei* ϵ for this purpose was indicated by the results of preliminary experiments as well as by the theoretical considerations already mentioned. The results of one of these experiments concerning the response to L-leucine obtained by a compound microbiological method employing *Lactobacillus arabinosus* 17-5 as the primary assay organism is given in Fig. 3. The average magnification achieved over the linear portion of this response curve may be calculated to be 1,250,000 times, a value nearly 5 times that attained by the present arginine method and more than 1000 times that realized with the standard *L. arabinosus* assay of L-leucine.

Summary. It has been shown that the sensitivity of a microbiological assay method may be compounded by employing a secondary microbiological assay to determine the D-lactic acid component of the products obtained from the response of the original assay. Sensitivities of up to several thousand times those realized by the standard microbiological procedures have been shown to be possible theoretically in compound assays. The method described for the compound assay of arginine, designed purposely to be of relatively low sensitivity, was approximately 144 times as sensitive as the standard microbiological method for this amino acid. The average percentages of arginine found in casein (3.49%), gelatin (9.04%), and silk fibroin (1.02%), respectively, by the compound assay were found to be approximately the same as those (3.67%, 8.78%, and 1.02%,

respectively) by the standard method, although the probable errors ranged from 3.2% to 6.1% for the individual assays by the former method compared to 0.7% to 1.2% for those made by the latter method. The sample weights required per assay (each including triplicate determinations at each of 4 or 5 sample levels) were: casein 45 μg, gelatin 18 μg, and silk fibroin 142 μg. One hundred times these amounts were required for each assay by the standard microbiological method.

1. Camien, M. N., and Dunn, M. S., *J. Biol. Chem.*, 1953, v201, 621.

2. Dunn, M. S., Camien, M. N., Malin, R. B, Murphy, E. A., and Reiner, P. J., *Univ. Calif. Publ. Physiol.*, 1949, v8, 293.

3. Sarlet, H., Duchâteau, Gh., Camien, M. N., and Florkin, M., *Biochim. et Biophys. Acta*, 1952, v8, 571.

4. Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, 1944, v155, 591.

5. Horn, M. J., Jones, D. B., and Blum, E., *ibid.*, 1948, v176, 59.

6. Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods,* C. C. Thomas, Springfield, Ill., 1951, 489.

Received January 4, 1954. P.S.E.B.M., 1954, v85.

Recovery of New Agent from Patients with Acute Respiratory Illness.

(20825)

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An epidemic of acute respiratory illness occurred among the service personnel at Fort Leonard Wood, Missouri during the winter of 1952-1953(1). Influenza A′ virus was of etiologic importance in a majority of the cases during the mid-portion of the epidemic but not among the cases at the beginning or end of the outbreak. Approximately 20% of the non-influenzal patients presented clinical and X ray findings which were consistent with the diagnosis of primary atypical pneumonia (PAP) and many had acute respiratory disease (ARD)(2-5). However, in individual instances, it was difficult if not impossible to establish the etiology until the results of laboratory tests became available.

The present report is concerned with the isolation of a microbial agent, presumably viral in nature, which appears to have been of etiologic importance in the disease suffered by certain of the non-influenzal cases in the epidemic.

Materials and methods. Tissue culture. Tube and bottle cultures of HeLa cells[†] (human epidermoid carcinoma) were prepared by the general method of Scherer, Syverton, and Gey(6) except that tryptic digestion was not employed. The bottle (32 oz prescription size) and tube (16 x 150 mm) cultures were fed with nutrient fluid consisting of pooled human serum, 25 parts; chick embryo extract, 7 parts (2 parts 50% chick embryo extract and 5 parts 50% chick embryo extract ultrafiltrate); Hanks balanced salt solution, 68 parts, plus sufficient penicillin and streptomycin to give a final concentration of 50 units of each per ml. In order to remove the human serum contained in the nutrient fluid, the contents of the bottles and tubes were washed 3 times, immediately before use, with 20 or 1.0 ml, respectively, of 5% chicken serum in Hanks-Simms solution containing antibiotics. Finally, 30 ml (bottles) or 0.6 ml (tubes) of maintenance solution (10 parts pooled chicken serum; 5 parts 50% chick embryo extract ultrafiltrate; 85 parts Hanks balanced salt solution and antibiotics) were added to the

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[*] The technical assistance of Vincent V. Hamparian, Sgt., U. S. A. is gratefully acknowledged.

[†] Bottle and tube cultures of HeLa cells and the nutrient and maintenance solutions were obtained from Microbiological Associates, Inc., Bethesda, Md.

washed cultures. At time of use, the bottle cultures contained about 6-10 million cells and were 3-5 days old; the tube cultures contained about 60-80 thousand cells and were 1-3 days old. The bottles and tubes were kept tightly stoppered prior to and following inoculation with the material under study and were incubated at 36°C in a stationary position.

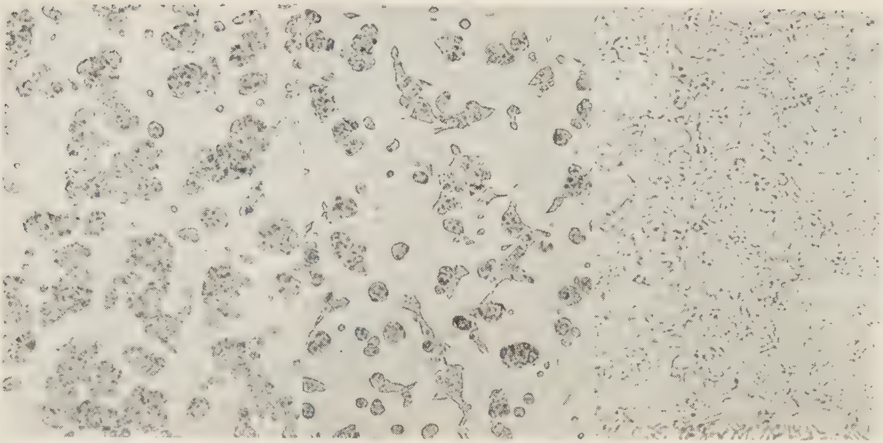
Isolation of strains of respiratory illness (RI) agents. Throat washings (collected in veal infusion broth and stored frozen at –70°C) from a patient with PAP (case 67) in the epidemic were centrifuged briefly to remove gross particles and then inoculated into roller tube cultures of adult human tracheal epithelium prepared by the general method of Enders(7). A cytopathogenic agent (RI-67) was recovered in first passage in such tissue and this organism was readily propagated in HeLa cells following 3 additional passages in tracheal cultures. All further isolations were attempted by direct inoculation into the HeLa culture medium.

For subsequent isolation attempts, the throat washings from 29 patients in the epidemic collected within the first 2 days of illness were inoculated in 0.1 ml amount into the HeLa cell culture tubes. If no cytopathogenic effect was observed within 3 days, the tissues and fluid were ground in a mortar and passed to new tubes. Cultures failing to show typical degeneration in 2 or 3 passages were considered negative and were discarded. Uninoculated control tubes were always included for comparison. Four cytopathogenic agents (in addition to the agent from patient 67 which was re-isolated directly in HeLa culture) were recovered from 2 patients with PAP and from 2 others with ARD. These agents appeared essentially identical biologically except for a minor antigenic heterogeneity detectable in neutralization tests with sera from patients convalescent from the disease. In this report, only the results of studies with the RI-67 agent are presented.

Serum neutralization test. 1. Seed virus. Flask cultures inoculated with 5.0 ml of passage virus titering 10^{-1} or 10^{-2} usually showed complete degeneration after 48 hours incubation. The cells and fluid were harvested at this time, homogenized in a Waring Blendor and the centrifuged supernate (2000 rpm for 10 minutes) which represented the seed virus was preserved at –70°C in glass-sealed ampoules. This material, titrated in HeLa cell cultures, caused significant degeneration within 2 days when diluted 10^{-1} or 10^{-2} (0.1 ml inoculum dose). *2. Neutralization procedure.* To serial 4-fold dilutions of patient's serum (inactivated at 56°C for 30 minutes) in 0.15 ml volume were added 0.3 ml amounts of seed virus diluted to contain about 10 cytopathogenic doses (strong or complete degeneration). Following incubation at 36°C for 30 minutes, pairs of tubes were inoculated with 0.15 ml amounts of each mixture. The cultures were observed for 2 or 3 days (until complete degeneration was noted in tubes in which antibody was absent) and the neutralization titer of the serum was considered to be the highest initial dilution of the serum which completely inhibited cell degeneration. Uninoculated control tubes and virus titrations were always included in the tests. Acute and convalescent sera from the same patient were tested simultaneously.

Complement-fixation (CF) test. The seed virus preparations described above were satisfactory complement-fixing antigens; uninoculated cultures, treated in the same manner, provided control antigen. The CF procedure was essentially the same as routinely employed in this laboratory for Q fever(8). The patient's serum was inactivated at 56°C for 30 minutes and 0.25 ml amounts of serial 2-fold dilutions of the serum, covering the range from 1:5-1:160, were allowed to react with 2 exact units of guinea pig complement in 0.5 ml volume and 2 units of viral antigen in 0.25 ml amount or 0.25 ml of normal control antigen in the same dilution. After incubation at 37°C for 1 hour, 0.5 ml of the hemolytic system, which consisted of a mixture of 0.25 ml of 3% sheep cell suspension and 0.25 ml of physiological saline solution containing 3 units of sheep cell amboceptor, were added and the tests were read after final incubation at 37°C for 30 minutes. The titer of the serum was considered to be the highest initial dilution of serum causing 3+ or greater fixation of complement.

Miscellaneous tests. The methods for influenza virus isolation and for the diagnostic



Complete degeneration Moderate degeneration No degeneration (control)

FIG. 1. Cytopathogenic effect of RI-67 agent for HeLa cells 2 days after inoculation, wet preparation, X70.

hemagglutination-inhibition test with cholera filtrate treated sera have already been described(9). Tests for Q fever were performed as described by Robbins *et al.*(8) and the CF test for psittacosis-lymphogranuloma venereum group infection using phenol-enhanced antigen(10) was conducted by a modification of the same general method. Cold agglutination tests were performed by the standardized procedure used in this laboratory(11) and the *Streptococcus MG* agglutination technic of Thomas *et al.*(12) was employed.

Results. Properties of the RI-67 agent. Cytopathology. Inoculation of the RI-67 agent, obtained from throat washings of patient 67, into HeLa culture tubes usually produced visible degenerative changes in cells in 24 hours and complete destruction in 48 hours. Fig. 1 shows the appearance of uninoculated culture cells and of culture cells showing moderate and complete degeneration viewed in the wet preparation. In the course of degeneration the cells lose their processes, become globoid and aggregate loosely. This is followed by accumulation of compact masses of darkly granular cells of decreased size and spherical shape. The cell surfaces may be irregular and covered with small globoid or vermiform extrusions.

Infected cells grown on coverslips, when fixed briefly with Zenker's-acetic solution and stained by the haematoxylin-eosin or Giemsa method, revealed rounding and clumping and formation of extrusions from the cell surface similar to that observed in wet preparations of the same material. In addition, intense basophilia of the nucleus and cytoplasm was observed and nuclear degeneration characterized by margination of chromatin, increased granularity, pyknosis or reticulum formation with apparent vacuolation was present. Occasionally, uninoculated cells which presented a darkened cytoplasm and appeared to be spontaneously degenerated, showed extrusions from the cell surface similar to those observed in the infected cells. No cytoplasmic or intranuclear inclusions similar to those seen in certain viral infections were observed in cells infected with RI-67 agent.

Attempt to cultivate RI-67 agent in animals and eggs. RI-67 tissue culture seed titering 10^{-2} was inoculated into 1-day-old baby mice and 5-day-old baby hamsters by the intranasal, intraperitoneal and intracerebral routes. In addition, 12-14 g mice were injected intraperitoneally and intracerebrally, and young adult guinea pigs were inoculated intraperitoneally. None of the animals inoculated intraperitoneally or intracerebrally developed signs of overt illness within 21 days following inoculation and the baby mice and hamsters inoculated intranasally did not show lesions in their thoracic or abdominal viscera when sacrificed on the fifth or eleventh day following inoculation. Passage of the agent to 8-10 g mice by the intranasal route produced no

TABLE I. Result of Neutralization and Complement-Fixation Tests with RI-67 Agent and Sera from Individual PAP, ARD and Influenza Cases, Fort Leonard Wood Epidemic.

		Serum		Neutralization test result						
				Serum dilution						
Patient	Clinical diagnosis	Date	Day dis.	1:2	1:8	1:32	1:128	1:512	Titer	C.F. titer
#67	PAP	1/ 5/53	2	4+[*]	4+	4+			<1:2	<1:5 [†]
(RI-67)		1/17/53	14							1:5
		2/ 2/53	30	0	0	0	2+	4+	1:32	1:40
#37	PAP	1/ 3/53	3	4+	4+	4+	4+	4+	<1:2	<1:5
		1/13/53	13	0	0	3+	4+	4+	1:8	1:40
		2/ 2/53	33	0	0	0	0	3+	1:128	1:80
#34	ARD	1/ 3/53	2	4+	4+	4+			<1:2	<1:5
		1/13/53	12							1:40
		2/ 4/53	34	0	0	0	0	3+	1:128	1:80
#13	Influenza	12/18/52	1	0	2+	4+			1:2	<1:5
	A′	1/ 9/53	22	0	2+	4+			1:2	<1:5

[*] Numbers indicate degree of tissue degeneration. 4+, complete; 3+, strong; 2+, moderate; 1+, slight degeneration. The table shows the result obtained in a single tube of the pair; the duplicate tubes in the series showed essentially identical findings. Control tests with serum alone did not show degeneration.

[†] None of the sera was anticomplementary and none reacted with normal control antigen.

changes which could not also be found in uninoculated mice from the same colony.

Six-day embryonated eggs inoculated via the yolk sac and 11-day eggs injected into the allantoic or amniotic cavity with the RI-67 agent showed no evidence of pathological effects following incubation at 35°C for 3 days. The allantoic and amniotic fluids as well as 20% suspensions of yolk sac and chick embryo harvested from these eggs failed to cause specific degeneration on inoculation into HeLa cultures. Furthermore, none of the allantoic or amniotic fluids agglutinated chicken or human "O" red blood cells when incubated together at 20° or 4°C.

Attempt to cultivate RI-67 agent on artificial media. HeLa culture preparation of RI-67 agent which titered 10^{-2} in HeLa tubes was inoculated on blood agar, 20% ascitic fluid agar, 10% rabbit serum-veal broth and Brewer's thioglycolate medium and incubated aerobically and anaerobically at 36°C. None of the cultures showed growth in 9 days and the ascitic agar failed to show pleuropneumonia organisms when examined[‡] by the agar stain method of Dienes(13).

Serological reactions with RI-67 agent and specimens from Fort Leonard Wood cases. The results of neutralization and complement-fixation tests performed with sera from representative patients in the epidemic is shown in Table I. It is seen that the neutralizing antibody titer for the RI-67 agent rose from <1:2 to as high as 1:128 in the 3 illustrative cases of PAP and ARD while sera from the influenza patient failed to show such increase. The complement-fixation titers similarly rose from <1:5 to 1:40 or 1:80 in the patients with PAP and ARD but not with influenza.

In the group of cases listed in Tables I and II, the maximum neutralizing antibody levels against the RI-67 agent were usually attained after the 21st-30th day of the disease while maximum levels of CF antibody were generally reached by the end of the second week (case No. 67 from which the agent was isolated was the only exception found to date).

Table II summarizes the results of neutralization and complement-fixation tests performed with the sera from a representative group of patients in the epidemic and the RI-67 agent. It is seen that 12 of 14 persons with PAP or ARD showed positive findings in neutralization tests for RI-67 agent and 13/14 were positive by CF; none of the influenza cases was positive.

The cases which showed positive results with RI-67 agent gave negative findings in tests for influenza A, B and C, and for cold and *Streptococcus MG* agglutinins. Likewise, none of the sera tested showed CF antibodies

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[‡] We are indebted to Dr. R. G. Wittler for these examinations.

TABLE II. Serological Findings in Representative Group of Cases from the Fort Leonard Wood Epidemic.

		No. cases showing positive[*] findings						
		RI-67 agent		Influenza HI				
Diagnosis	Total No. of cases	Neut.	C.F.	A′ FW-1-50	B-Lee	C-1233	Cold agglut.	Strept. MG
PAP	7	6(32X)[†]	7(16X)[†]	0	0	0	0[‡]	0[‡]
ARD	7	6(16X)	6(8X)	0	0	0	0	0
Influenza	5	0(0X)	0(0X)	5	0	0	0	0

[*] A positive result was a 4-fold or greater increase in antibody titer.

[†] Numbers in parentheses are the fold-increase (geometric mean) in serum titer of the paired specimens in the group.

[‡] None of the *Streptococcus MG* agglutination titers of individual specimens exceeded 1:8. The cold agglutination titers were all <1:4.

for Q fever or psittacosis-lymphogranuloma venereum group antigen.[§]

Serologic reaction of RI-67 agent with sera from other human diseases and from hyperimmunized animals. Neutralization tests performed with RI-67 agent and paired sera from proved cases (showing a 4-fold or greater increase in antibody titer to the responsible agent) of human influenza A′ (1 case in addition to those shown in Table II) influenza B (1 case), psittacosis (2 cases), pigeon ornithosis (1 case), Q fever (2 cases) all failed to show a rise in antibody titer for RI-67. Similar negative results were also obtained with the paired sera[||] from 3 patients with common cold and from 4 cases of primary atypical pneumonia (Cleveland, Ohio 1948 and 1953) which showed significant increase in cold or *Streptococcus MG* agglutinin. Appropriate specimens from serologically proved cases of LCM aseptic meningitis (1 case), mumps meningitis (2 cases), herpes meningitis (2 cases) and from clinical cases of infectious mononucleosis and varicella (1 each) also failed to show increase in neutralizing antibody for RI-67 agent in the convalescent specimen.

Despite this failure to obtain an antibody rise in these paired specimens, almost half (10/22) were found to have a detectable level of RI-67 neutralizing antibody which was present at a constant level (1:2-1:32) in both sera.

Further evidence for lack of relationship of RI-67 to a number of viral and rickettsial agents was obtained in tests with hyperimmune animal sera. Sera against influenza A′ (Strain A-FLW-1-52, chicken), influenza B (Motulsky-1952, chicken), influenza C (1233, chicken), ornithosis (MP-F97, chicken), Q fever (Nine Mile, guinea pig), LCM (rabbit), herpes (Laski, rabbit) and Newcastle Disease virus[¶] (chicken) failed to neutralize RI-67 in a serum dilution of 1:2 or greater.

Discussion. It appears that the RI-67 virus recovered from a case of primary atypical pneumonia in the epidemic at Fort Leonard Wood is unrelated to the agents which cause Q fever, psittacosis and influenza. The possible relationship of RI-67 to any of the various infectious agents recovered during the past decade from animals, eggs, or tissue cultures inoculated with materials from cases of the common cold(5,14,15) or primary atypical pneumonia(5,14) remains to be determined.

The consistent occurrence of increase in antibody titer against RI-67 in convalescent sera from patients with PAP and ARD in the epidemic suggests that the new agent may be of etiological significance at least in certain outbreaks of these diseases.

Summary. A microbial agent, presumably a virus, was recovered from throat washings from a patient with primary atypical pneumonia in an epidemic of acute respiratory illness which occurred at Fort Leonard Wood during the winter of 1952-1953. This agent multiplies in human cell tissue cultures producing obvious cytopathogenic changes but not in common laboratory hosts. The patient whose throat washings yielded the virus developed specific neutralizing and complement-fixing antibodies for the agent. Other patients in the epidemic with primary atypical pneumonia (PAP) or undifferentiated acute respiratory disease (ARD) also developed antibodies for the agent but those cases with proved influenza A′ did not. A portion of the population maintains an antibody level against the new agent suggesting a rather general experience with it.

[§] These tests were performed by A. Bankhead and O. W. Dandridge.

[||] We are indebted to Dr. W. S. Jordan, Jr. for these specimens.

[¶] This was kindly furnished by the Bureau of Animal Industry, U. S. Dept. of Agriculture.

Confirmatory experiments. Certain of the basic observations presented in this report have been repeated by one of the authors (M.R.H.) in the laboratory of Dr. John H. Dingle at Western Reserve University. The RI-67 agent was taken to Dr. Dingle's laboratory and the cytopathogenic effect for HeLa cells was demonstrated in cultures prepared at Western Reserve University as well as in cultures taken from this laboratory. Neutralization tests with known positive and negative paired sera were also carried out using the cultures and reagents from the 2 sources and essentially identical results were obtained.

Addendum. Since the present manuscript was submitted for publication, Rowe et al. (PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 570) *reported the recovery of an agent cytopathogenic for HeLa cells from cultures of human adenoid tissue. Tests performed in our laboratory and that of Dr. Huebner gave presumptive evidence of an immunological relationship between the RI-67 and the adenoid degenerative agent.*

1. Hilleman, M. R., and Adair, C. V., to be published.
2. U. S. Army Commission on Acute Respiratory Diseases, *Am. J. Pub. Health,* 1944, v34, 347.
3. ———, *ibid.,* 1946, v36, 439.
4. ———, *Medicine,* 1947, v26, 441.
5. Stuart-Harris, C. H., *Influenza and Other Virus Infections of the Respiratory Tract,* Edward Arnold and Co., London, 1953, 235 p.
6. Scherer, W. F., Syverton, J. T., and Gey, G. P., *J. Exp. Med.,* 1953, v97, 695.
7. Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 100.
8. Robbins, F. C., Rustigian, R., Snyder, M. J., and Smadel, J. E., *Am. J. Hyg.,* 1946, v44, 51.
9. Hilleman, M. R., and Werner, J. H., *J. Immunol.,* 1953, v71, 110.
10. Nigg, C., Hilleman, M. R., and Bowser, B. M., *J. Immunol.,* 1946, v53, 259.
11. Smadel, J. E., *Viral and Rickettsial Infections of Man,* Ed. by T. M. Rivers, second edition, J. B. Lippincott Co., Philadelphia, 1952, p87.
12. Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E., Jr., and Horsfall, F. L., Jr., *J. Clin. Invest.,* 1945, v24, 227.
13. Dienes, L., *J. Infect. Dis.,* 1939, v65, 24.
14. Horsfall, F. L., Jr., *Viral and Rickettsial Infections of Man,* Ed. by T. M. Rivers, 2nd edition, J. B. Lippincott Co., Philadelphia, 1952, pp. 378-391.
15. Andrewes, C. H., Chaproniere, D. M., Gompels, A. E. H., Pereira, H. G., and Roden, A. T., 1953, *Lancet,* vII, 546.

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The Oxidation of Mannitol.* (20826)

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The value of mannitol, an isomer of sorbitol, as an adjunct to the diabetic diet has recently been reviewed by Olmsted(1). It was concluded that mannitol could not be of much importance as an adjunct to the diabetic diet, since 80% or more of the mannitol administered is excreted in the urine. These conclusions were apparently based on the data obtained by IV administration(2-6). Mannitol has also been known to form significant amounts of liver glycogen after oral administration(2,7), and under these conditions it is unlikely that mannitol can be excreted in the amounts indicated above. However, no posi-

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

TABLE I. Recovery of C^{14} after Administration of R.A. Mannitol.

	Hours of CO_2 collection	Experiment No. 1	2	3	4	5	6	7
		I.P. inj.		Oral feeding				Spleen injected
% administered radioactivity recovered in expired CO_2 (accumulative)	3	.4	0	3.5	.2	.5	.3	.5
	6	.8	.5	21	4	5	4	70
	9	1.1	1.7	35	11	13	12	71
	12	1.3	2.1	38	29	25	23	71
	18	1.6	2.4	46	42	54	47	71
	24	2.1	3.2	48	46	63	58	71
Urine		97	77	13	11	8	8	5
Stool						12	12	1
Total recovery		99	80	61	57	83	78	78
mg mannitol admin.		12	180	2	240	240	240	2
Animal wt, g		183	160	183	155	190	190	190

tive conclusions regarding the metabolic utilization of a substance can be obtained from liver glycogen data; for this, labeled material must be used. The differences in regard to the utilization of the sugar alcohol may be due to the technics employed in the administration. In order to clarify the overall metabolic fate of mannitol, we have studied its rate of oxidation and excretion after oral and IP administration. Mannitol uniformly labeled with C^{14} was employed.

Methods. Preparation of radioactive mannitol. Mannitol C^{14} was prepared by the catalytic reduction of C^{14} uniformly labeled glucose with Raney nickel in alkaline solution. The procedure is similar to that used for the preparation of sorbitol(8). The mannitol was purified by a series of crystallizations from water and finally from aqueous ethyl alcohol. The product was identified as mannitol by its M.P. (162°-163°C) and mixed M.P. (163°-164°C) with an authentic mannitol sample (164°C). In addition, the specific activity of a mixture of labeled mannitol and carrier mannitol did not change on repeated crystallization. *Animal treatment.* Nonfasted male albino rats were used. The mannitol was given intraperitoneally, orally, and in one experiment directly into the portal system by way of the spleen. For the latter experiment the spleen was exteriorized the day before the experiment. The methods used for collecting the expired CO_2 and for measurement of radioactivity are the same as that used for sorbitol(8).

Results and discussion. The data given in Table I show that mannitol, when given parenterally, is excreted to a large extent in the urine, which is in agreement with numerous previous reports. The high urinary excretion after parenteral injection is balanced by a low rate of oxidation of about 2-3%. On the other hand, oral feeding of the sugar alcohol leads to oxidation values of about 50% of the injected radioactivity. These divergent results regarding mannitol utilization, obtained by the two methods of administration, indicate either that the mannitol is oxidized, initially at least, only by the liver or that mannitol is acted on by the intestinal flora before absorption from the intestinal lumen. In the latter case the metabolic utilization of fed mannitol is due to the absorption of conversion products of bacterial action. In order to differentiate between these two possibilities, mannitol was injected directly into the portal system by way of the spleen. In this experiment (Exp. 7), 71% of the mannitol was oxidized, indicating that the liver is the most likely site of mannitol oxidation and that bacterial action in the intestinal tract does not play a measurable role in the oxidation. One must assume from these results that mannitol cannot be oxidized by tissues other than the liver and also that the kidneys have a low renal threshold for the sugar alcohol. In parenteral injection a large fraction of the material is subjected largely to the excretory action of the kidneys before it is made available to the liver, and it is rapidly excreted, due to the low renal threshold, before it can be oxidized. In oral feeding practically all of the material must pass first through the liver before it is put into the general circulation; hence the oxidation values of 48-63% are obtained. It is of interest to note that Dominguez, Corcoran and Page(4) in evaluating the use of mannitol in renal function concluded from their studies that the utilization of mannitol in humans, although small after IV injection, is presumably metabolic.

Although metabolites are routinely given parenterally in many experimental studies in order to by-pass the intestine, it is obvious from the results reported here that data obtained from parenteral injection should be examined with comparable data obtained after oral administration for a true evaluation of the metabolic pathway.

Summary. Radioactive mannitol has been administered to rats with the following results: 1. When given orally, about 50% of the mannitol carbon is recovered in the expired CO_2 in 12 hours. 2. With intraperitoneal injection, the mannitol is largely recovered in urine with only 2-3% of the mannitol carbon recovered in the expired CO_2. 3. By injection directly into the portal system by way of the spleen, oxidation comparable to or greater than that obtained by oral administration is obtained. It is concluded from these results that mannitol can be oxidized by the body if it is administered by a route that makes it first available to the liver, as in oral feedings.

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1. Olmsted, William H., *Diabetes,* 1953, v2, 132.
2. Todd, W. R., Meyers, Jane, West, E. S., *J. Biol. Chem.,* 1939, v127, 275.
3. Smith, W. W., Finkelstein, Norma, Smith, H. W., *ibid.,* 1940, v135, 231.
4. Dominguez, R., Corcoran, A. C., and Page, I. H., *J. Lab. and Clin. Med.,* 1947, v32, 1192.
5. Elkinton, J. R., *J. Clin. Invest.,* 1947, v26, 1088.
6. Clark, J. K., and Barker, H. C., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 152.
7. Carr, C. J., and Krantz, J. C., *J. Biol. Chem.,* 1938, v124, 221.
8. Wick, Arne N., Almen, Mary C., and Joseph, Lionel, *J. Am. Pharm. Assn.,* 1951, v40, 542.

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Urinary Oxalate Excretion by Man Following Ascorbic Acid Ingestion.* (20827)

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Oxalic acid has long been known to be a product of the *in vitro* oxidation of ascorbic acid(1-5). *In vivo,* Scheinkman(6) showed that ascorbic acid ingestion by guinea pigs resulted in increased urinary oxalate. Burns *et al.*(7) found that one of the end products of the metabolism of radioactive 1-C^{14}-L-ascorbic acid administered to guinea pigs was urinary oxalate. Since large doses of ascorbic acid are being prescribed by physicians(8,9), an investigation of the effect of ascorbic acid on the urinary oxalate excretion of man is warranted. This study is concerned with that investigation.

Procedure. In the first experiment, 11 healthy adult males on their regular diet collected 24 hour control urine samples. Each subject then ingested, in addition to his regular diet, 3 g of pure ascorbic acid at each mealtime (9 g/day) for 2 days, collected a 24 hour urine sample on the second day and another 24 hour control urine sample one week later. The results indicated that there was increased oxalate excretion with the daily ingestion of 9 g of extra-dietary ascorbic acid. A second experiment was conducted to determine at what minimum dosage of supplementary ingested ascorbic acid an increase in urinary oxalate occurred. Forty adult males were placed into groups ingesting different amounts of ascorbic acid ranging from 0.25 g to 8 g (divided into 3 doses as in the first experiment) daily for one week at the end of

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* Supported in part by a grant from Hoffmann-LaRoche Inc., Nutley, N. J.

which each member collected a 24 hour urine sample. All subjects collected 24 hour control urine samples prior to receiving extra-dietary ascorbic acid and at the end of the first and second weeks following cessation of the ingestion of extra-dietary ascorbic acid. The groups taking 0.25, 0.50, and 2 g of ascorbic acid daily each consisted of 7 subjects. The groups ingesting 4 and 8 g of ascorbic acid each consisted of 6 subjects. The method for determining urinary oxalate was essentially that of Powers and Levatin(10). Care was taken to prevent the *in vitro* conversion of urinary ascorbic acid to oxalic acid. Urine was voided into a large bottle containing 225 ml of stabilizing solution (20% metaphosphoric acid and 3.3% thiourea). In order to test the procedure, 9 g of ascorbic acid were added to a composite urine sample containing the stabilizing solution. This preparation was then allowed to stand 24 hours at room temperature during which time the oxalate content increased only 1.2 mg (as oxalic acid) which amount was within the experimental error of the method. All urine samples were analyzed individually. The values for the oxalate content of the control urines for each subject were averaged. This average was subtracted from the value for the urinary oxalate excretion of the subject following ascorbic acid ingestion to determine the change, whether positive, negative, or zero.

Results. The mean oxalic acid excretion of 51 males without extra-dietary ascorbic acid was 38.3 ± 1.7 mg (standard error). This value was based on 142 determinations. The range of urinary oxalate output among the controls was 16 to 64 mg oxalic acid. Values reported in the literature for the normal 24 hour urinary oxalate excretion of man range from 14.0 to 56.0 mg with averages of 30 to 35 mg oxalic acid(2,11).

The remarkable constancy of the oxalic acid excretion for an individual on his regular diet without supplementary ascorbic acid is indicated by the average of the maximum variation among controls for each individual which was only 5.2 mg oxalic acid. The greatest variation among controls for an individual was 19 mg.

The effect of ingested ascorbic acid on urinary oxalate excretion is shown in Fig. 1 in which the increase in 24 hour urinary oxalic acid in mg is plotted against ascorbic acid ingested daily in grams. The ingestion of 4 g of ascorbic acid results in an average increase of 12 mg of excreted oxalic acid significant at the 6% level of probability; 8 g, an average increase of 45 mg of oxalic acid, and 9 g, an average increase of 68 mg of oxalic acid, these last two increases being statistically highly significant. The ingestion of less than 4 g of ascorbic acid daily resulted in a negligible increase in urinary oxalate.

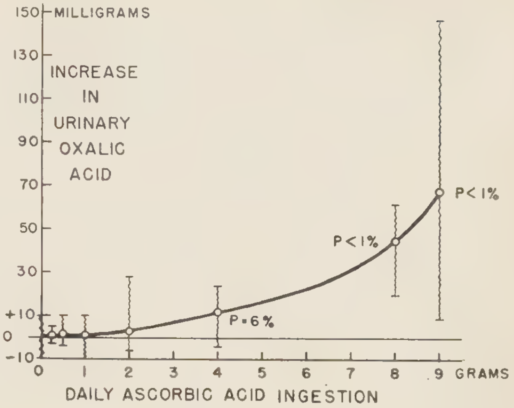

FIG. 1. Effect of amount of ascorbic acid ingested daily on increase in urinary oxalate (as oxalic acid). Circles on curve represent avg increase in excretion of urinary oxalic acid for each group. "Increase" in urinary oxalic acid is the difference between 24 hr urinary oxalic acid excretion of an individual following ingestion of ascorbic acid and avg of the values of his control urines collected when he received no extra-dietary ascorbic acid. The 0.25, 0.50, 1 and 2 g groups consisted of 7 subjects; 4 and 8 g groups, 6 subjects, and 9 g group, 11 subjects. Vertical wavy lines indicate maximum and minimum individual increases (sometimes negative) found within each group except in the case of controls (0 g extra-dietary ascorbic acid ingested) where they indicate the greatest variation between controls of a single individual in the whole study. P = probability level.

Further investigation is being conducted on laboratory animals to determine whether urinary oxalate calculi and kidney changes may be a consequence of massive ascorbic acid ingestion over a long period of time.

Summary. An investigation was conducted to determine the urinary oxalate excretion of normal males on their regular diet, and to determine to what extent extra-dietary ascorbic acid would increase this excretion. The mean 24 hour urinary oxalic acid excretion of

51 healthy adult males on their regular diets was 38.3 ± 1.7 mg. The 24 hour excretion of any individual was remarkably constant over a three week period. The daily ingestion of 4 or more g of ascorbic acid increased urinary excretion of oxalic acid; whereas the daily ingestion of less than 4 g of ascorbic acid produced no significant increase in oxalate excretion.

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1. Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W., and Smith, F., *J. Chem. Soc.*, 1933, 1270.

2. Flaschenträger, B., and Müller, P. B., *Z. Physiol. Chem.*, 1938, v251, 52.

3. Ghosh, J. C., and Rakshit, P. C., *Biochem. Z.*, 1938, v299, 394.

4. Jurist, A. E., and Christiansen, W. J., *Am. J. Pharm.*, 1939, v111, 347.

5. Rosenfeld, B., *J. Biol. Chem.*, 1943, v150, 281.

6. Scheinkman, E. A., *Biochem. J.*, Ukraine, 1940, v16, 397.

7. Burns, J. J., Burch, H. B., and King, C. G., *J. Biol. Chem.*, 1951, v191, 501.

8. Lowry, O. H., Bessey, O. A., and Burch, H. B., Proc. Soc. Exp. Biol. and Med., 1952, v80, 361.

9. Massell, B. F., Warren, J. E., Patterson, P. R., and Lehmus, H. J., *New Eng. J. Med.*, 1950, v242, 614.

10. Powers, H. H., and Levatin, P., *J. Biol. Chem.*, 1944, v154, 207.

11. Jeghers, H., and Murphy, R., *New Eng. J. Med.*, 1945, v233, 208, 238.

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An Antipyretic Action of Aureomycin. Inhibition of Febrile Response to Influenza Virus in Rabbits.* (20828)

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Aureomycin has been reported to influence favorably the course of certain viral infections in man(1-3). Except for the psittacosis-lymphogranuloma group(4), however, there is no evidence that this drug either limits viral multiplication(5) or prevents production of lesions by these agents(6). It seemed possible, therefore, that the effect of aureomycin in these instances might be non-specific; that is, its action might be mediated through a mechanism other than one which influences the pathogenesis of the disease. For example, in those circumstances in which the major objective evidence for the effectiveness of aureomycin is provided by an early return of temperature to normal, the question of an antipyretic action may be raised. As an approach to this problem, an investigation was carried out to determine whether aureomycin could inhibit the pyrogenic response to a viral agent in a situation where infection was not concerned. The experimental model chosen for study was the febrile reaction produced in rabbits by intravenous inoculation of influenza virus(7).

Materials and methods. Virus. The virus employed was influenza B, Lee strain, which was propagated in the allantoic sac of chick embryos 9 to 11 days of age. Methods of cultivation were identical to those previously described(8). Infected allantoic fluids were stored at −30°C(9). Fluids were clarified by centrifugation at 3000 RPM for 10 minutes at 4°C and tested for bacteriological sterility on blood agar plates at 37°C for 24 hours. All virus infected allantoic fluids employed had hemagglutination titers of 1:512, using methods previously described(8). *Normal allantoic fluid.* Allantoic fluid was obtained from chick embryos handled in the same manner as described above except that they were not inoculated with virus. *Aureomycin.* Each injection of aureomycin consisted of 100

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* This investigation was conducted under the sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, and was supported in part by the Office of The Surgeon General, Department of the Army, and by grants from the Brush Foundation and Mr. Philip R. Mather.

mg of aureomycin hydrochloride† dissolved in 10 cc of 1.95% l-leucine diluent. Aureomycin of a single lot number was employed. Care was taken that the drug was completely dissolved before it was injected since when visible suspended particles were present, intravenous injection resulted in fever. *Animals.* Adult male or female rabbits of mixed breeds, weighing 2-3 kg, were employed. The animals' temperatures were taken at various intervals for at least two days prior to using them in an experiment in order to accustom them to the procedure. They were kept in individual cages without access to food or water during experiments and were removed from the cages when temperatures were taken or injections given. Rabbits which had been given influenza virus were not used in subsequent experiments for at least 4 weeks in order to avoid the period of resistance to influenza virus pyrogen which is present for 2 weeks after inoculation(10). *Design of experiments.* Four groups of 3-6 rabbits each were employed in each experiment. Rabbits of 2 groups were inoculated intravenously with 5 ml of allantoic fluid infected with influenza virus. Animals of the other 2 groups each received 5 ml of normal allantoic fluid intravenously. One of the groups inoculated with virus and one inoculated with normal allantoic fluid were given aureomycin either intravenously or intraperitoneally; the other 2 groups received 10 ml of l-leucine diluent by the same route. Aureomycin or diluent was administered at various times before and after inoculation of virus in different experiments. A fifth group of rabbits which received no injections was employed in most experiments. *Rectal temperatures* were taken every 30-60 minutes for several hours before and six hours after inoculation of virus or normal allantoic fluid. Temperatures were recorded as changes from the initial value, and the mean change in temperature at each time interval was calculated for each group of rabbits. Experiments were conducted at room temperature.

Experimental. Effect of intraperitoneal aureomycin before and after virus. To determine whether or not aureomycin could alter the pyrogenic reaction to intravenous inoculation of influenza virus, 100 mg of the drug was injected intraperitoneally 2 hours before and 1½ hours after intravenous inoculation of virus. The results of a typical experiment are presented graphically in Fig. 1. Two similar experiments yielded entirely comparable results. Control rabbits given diluent intraperitoneally and virus intravenously exhibited a characteristic febrile response with a maximum mean temperature increase of 2.8°F 5 hours after injection of virus. This temperature elevation was blocked completely in rabbits given aureomycin. In addition to this antipyretic effect, all rabbits injected with aureomycin intraperitoneally showed a marked fall in rectal temperature which was of the same magnitude in animals given aureomycin and virus as in those given aureomycin and normal allantoic fluid. The similarity of these temperature curves suggested that the antipyretic effect did not depend quantitatively upon the hypothermic action of the drug administered intraperitoneally. Rabbits which received no injections had little change in rectal temperature. Aside from temperature differences, rabbits injected with aureomycin reacted and appeared no differently from those given diluent; there was no indication of any discomfort or untoward effect. Peritoneal cavities of rabbits autopsied ½ to 3 hours after intraperitoneal administration of either aureomycin or diluent showed no grossly discernible differences save for the occasional presence of some residual drug in those injected with aureomycin. Thus, there was nothing to suggest that the observed effects of intraperitoneal aureomycin were produced by central vasomotor collapse or local irritation resulting in a state of shock or altered reactivity.

Effect of intraperitoneal aureomycin after virus. The effect of aureomycin when administered after the pyrogenic reaction was evident was next determined. The results of an experiment in which aureomycin was injected intraperitoneally 1½ hours after virus are presented in Fig. 2. Temperatures of all rabbits were similar 1½ hours after influenza

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† The aureomycin was kindly furnished by Dr. James M. Ruegsegger of Lederle Laboratories, Pearl River, N. Y.

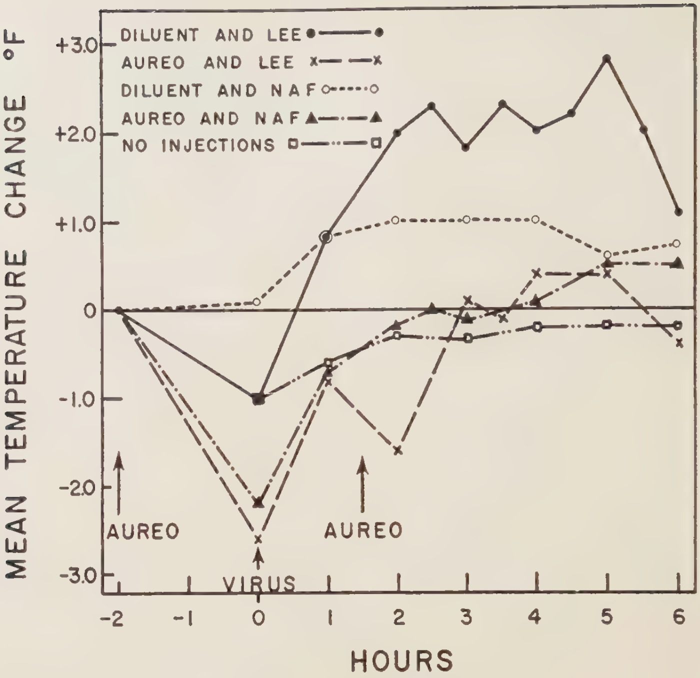

FIG. 1. Effect of intraperitoneal injection of 100 mg of aureomycin upon pyrogenic response of rabbits to intravenous inoculation of 5 ml of Lee virus. Aureomycin was injected 2 hr before and 1½ hr after virus. Controls injected with combinations of virus, diluent, normal allantoic fluid (NAF) and aureomycin are included.

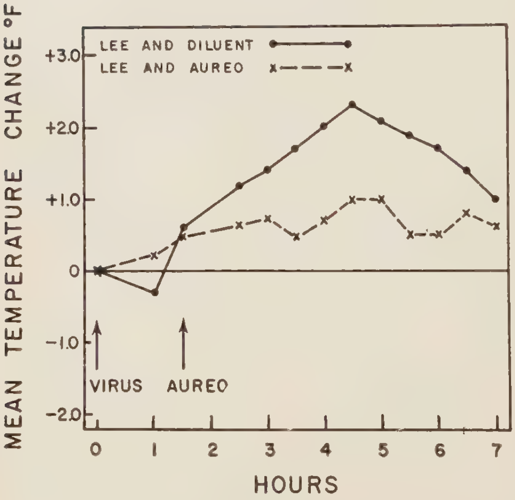

FIG. 2. Mean temperature change of rabbits injected intraperitoneally with 100 mg of aureomycin 1½ hr after intravenous inoculation of 5 ml of Lee virus.

B was inoculated, but animals given aureomycin at that time failed to develop the subsequent increase in fever shown by those which received diluent after virus. One similar experiment showed comparable results. These data indicate that aureomycin did exhibit antipyretic activity when administered after the pyrogenic response was established even though the temperature did not return to a normal value.

Effect of simultaneous intravenous inoculation of aureomycin and influenza B virus. To determine whether the hypothermic and antipyretic effects of aureomycin observed after its intraperitoneal injection depended in some specific manner upon the route of its administration rather than upon some pharmacologic property of the drug, other routes of injection were employed. Data from an experiment in which aureomycin was injected intravenously at the same time as virus are presented in Fig. 3. Two additional experiments conducted in the same manner yielded similar results. Aureomycin given intravenously prevented the febrile response of rabbits to influenza B virus. These data indicated that the antipyretic action of this drug was independent of the route of parenteral adminis-

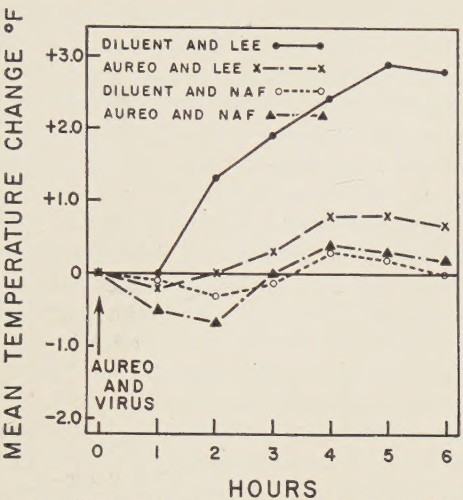

FIG. 3. Effect of intravenous injection of 100 mg of aureomycin upon febrile response of rabbits to intravenous inoculation of 5 ml of Lee virus.

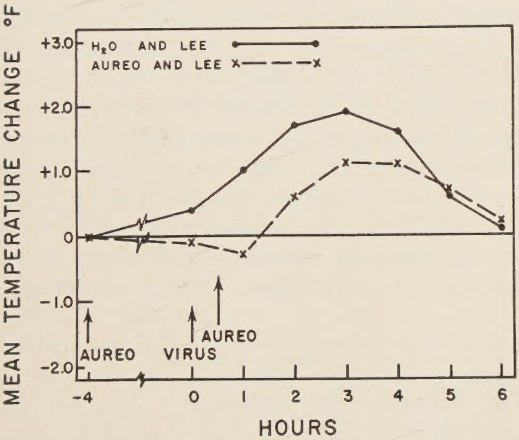

FIG. 4. Effect upon febrile response of rabbits given 1 g of aureomycin by gavage 4 hr before and ½ hr after intravenous inoculation of 5 ml of Lee virus.

tration. A hypothermic action of aureomycin was not evident by this route since there was little reduction of rectal temperatures below normal in rabbits given intravenous aureomycin and normal allantoic fluid. This observation gave additional evidence that the antipyretic activity was not a direct function of the hypothermic effect of the drug.

Effect of aureomycin by gavage before and after Lee virus. To determine the effect of oral aureomycin on the viral pyrogenic response, 1 g of aureomycin was given in 30 ml of water through a stomach tube 4 hours before and ½ hour after virus. The results of one experiment are presented in Fig. 4. The degree of fever produced in the treated rabbits was less than in controls. Four other similar experiments were done: in two the results were similar to those presented in Fig. 4, in one experiment no difference between aureomycin treated and control animals was apparent, and in one experiment the aureomycin treated animals developed more fever than controls. The marked variation in antipyretic effect achieved within the treated group in each experiment was responsible for failure to obtain reproducible results. This variability may have been the effect of variable absorption of the drug although experimental determinations to elucidate this possibility were not done. In order to reduce the mathematical effect of variation, geometric means were used to calculate temperature changes for all 5 gavage experiments combined. This procedure yielded temperature curves substantially identical to those presented in Fig. 4. However, since aureomycin was given at various times in different experiments these data could not be presented in a summarized form. Another difficulty encountered in gavage experiments was that the amount of fever produced in control animals was regularly less than that expected on the basis of previous experiments. Apparently the procedure of gavage itself reduced the pyrogenic response. In spite of these experimental difficulties, the trend was for aureomycin treated rabbits to manifest less fever than controls.

Effect of aureomycin on development of tolerance to the pyrogenic property of influenza virus. A febrile response to intravenous inoculation of influenza virus renders rabbits resistant to the pyrogenic action of the agent for a period of approximately 2 weeks(10). This tolerance is not related to immunity. To obtain evidence concerning the mechanism of the antipyretic action of aureomycin, experiments were designed to determine whether or not rabbits become resistant to the pyrogenic effect of influenza virus even if the febrile response were inhibited by the drug. Influenza B virus was inoculated intravenously into rabbits which

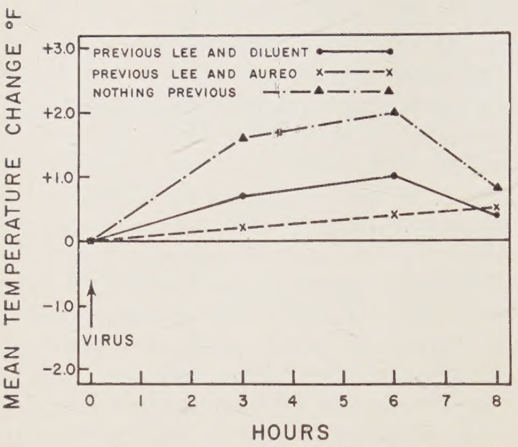

FIG. 5. Effect of antipyretic action of aureomycin upon acquisition of resistance to the pyrogenic property of influenza virus. Rabbits were inoculated intravenously with 5 ml of Lee virus 48 hr after intravenous inoculation of Lee virus and intraperitoneal injection of 100 mg of aureomycin or diluent.

received an intraperitoneal injection of aureomycin 2 hours before and a second injection 1½ hours after virus. The febrile response to virus was inhibited by aureomycin. Two days after the first viral inoculation these rabbits and controls which had been inoculated with virus and diluent received a second inoculation of virus. The results of this experiment are presented in Fig. 5. Rabbits inoculated with virus but in whom the occurrence of fever had been prevented by aureomycin behaved in the same manner toward subsequent inoculation of virus as those which had received virus and diluent and had experienced the febrile reaction. That is, both groups failed to develop fever. In this experiment temperatures were recorded only 0, 3, 6, and 8 hours after inoculation of virus so that peaks in the temperature curves may have been missed. At the times tested, however, refractoriness of rabbits to the pyrogen was apparent when compared to the response of animals which had not previously been inoculated with virus. These observations provided evidence that aureomycin like antipyrine(10) did not directly alter the host reaction to virus other than to inhibit the appearance of fever.

Effect of intraperitoneal aureomycin before or after typhoid vaccine. To determine whether aureomycin could exhibit antipyretic activity against a bacterial pyrogen, rabbits were inoculated intravenously with 1 ml of typhoid vaccine, containing 10^8 killed organisms. Aureomycin was injected intraperitoneally 2 hours before and 1 hour after vaccine in one group of rabbits and 1 hour after vaccine in another. The results of this experiment are shown in Fig. 6. Fever was markedly reduced in animals given aureomycin both before and after vaccine. When aureomycin was administered after the pyrogenic reaction was established, although an immediate reduction in temperature was observed and subsequent fever was less than that of controls, the febrile response was not entirely inhibited. The effect of aureomycin was not as marked as in experiments in which influenza virus was the pyrogenic stimulus, but this experiment does indicate that aureomycin can abolish or modify the reaction to a bacterial pyrogen.

Discussion. The original pharmacological investigation of aureomycin by Harned *et al.* (11) failed to disclose evidence of an antipyretic activity of this drug. Indeed, rabbits which were given 25 mg of aureomycin per kg intravenously 2 hours after intravenous inoculation of typhoid vaccine, experienced more fever than the controls. Since intravenous administration of some lots of aureomycin

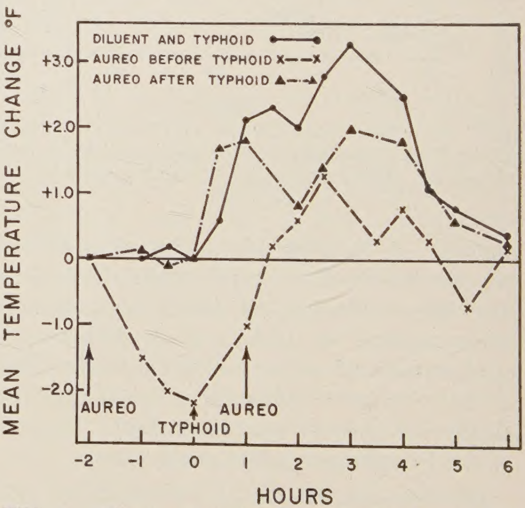

FIG. 6. Effect of intraperitoneal injection of 100 mg of aureomycin upon pyrogenic response of rabbits to intravenous injection of 1 ml of typhoid vaccine. Aureomycin was given 2 hr before and/or 1 hr after typhoid bacilli.

may produce fever, it seems possible that the preparation of aureomycin used in the experiments reported by these workers may have itself been pyrogenic, and thus obscured any antipyretic effect of the drug. Furthermore, the drug was given only after establishment of the pyrogenic reaction, a situation in which less antipyretic effect is observed.

Beeson(12) administered 2 g of aureomycin by gavage to 2 rabbits 2 hours before injection of typhoid vaccine intravenously and found no evidence of antipyretic activity of the drug. A similar lack of effect in individual rabbits given aureomycin by gavage was noted in the experiments reported in this paper although good protection was obtained in other rabbits. Consequently, this mode of administration of the drug cannot be considered the optimal one to demonstrate this property of aureomycin.

The pyrogenic effect of influenza virus is closely associated with its capacity to agglutinate erythrocytes but is unrelated to the infectivity of viral particles(9). Since viral multiplication does not occur in rabbits, the question of a chemotherapeutic action in this experimental model does not arise. The similarity of the effect of aureomycin to that of antipyrine against influenza virus pyrogen has been commented upon, and by analogy suggests a non-specific antipyretic action.

The experimental data presented do not permit conclusions concerning the mechanism of this action of the drug, but irrespective of mechanism, it is apparent that there are circumstances, however specialized these may be, in which aureomycin can exhibit an antipyretic effect. Certainly the evidence does not indicate whether or not this pharmacological property of aureomycin is capable of expression in any clinical situation. However, in the absence of direct evidence of a specific effect of aureomycin in viral diseases, the data do suggest that the possibility of an antipyretic action of this drug should receive consideration in the evaluation of its action.

Summary. Intraperitoneal or intravenous injection of aureomycin prevented the febrile reaction of rabbits to intravenous inoculation of influenza B virus, but administration of aureomycin by gavage did not consistently prevent fever. Although intraperitoneal injection of aureomycin reduced rectal temperature below normal, the data suggest that this hypothermic action of the drug was not responsible for the antipyretic effect observed. Aureomycin did not alter the development of tolerance to the pyrogen. Intraperitoneal aureomycin also modified the febrile response of rabbits to a bacterial pyrogen, typhoid bacilli.

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1. Collins, H. S., Wells, E. B., Goche, T. M., and Finland, M., *Am. J. Med.,* 1950, v8, 4.

2. Meikeljohn, G., and Shragg, R. I., *J.A.M.A.,* 1949, v140, 391.

3. Carter, H. C., and Sydenstricker, V. P., *Am. J. Med.,* 1951, v10, 309.

4. Wagner, J. C., Andrew, V. W., and Watson, M. S., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 79.

5. Wong, S. C., and Cox, H. R., *Ann. N. Y. Acad. Sc.,* 1948, v51, 290.

6. Kass, E. H., Lungren, M. M., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 520.

7. Wagner, R. R., Bennett, I. L., Jr., and LeQuire, V. S., *J. Exp. Med.,* 1949, v90, 321.

8. Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *J. Exp. Med.,* 1948, v87, 385.

9. Olitsky, P. K., Casals, J., Walker, D. L., Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Lab. and Clin. Med.,* 1949, v34, 1023.

10. Bennett, I. L., Jr., Wagner, R. R., and LeQuire, V. S., *J. Exp. Med.,* 1949, v90, 335.

11. Harned, B. K., Cunningham, R. W., Clark, M. C., Cosgrove, R., Hine, C. R., McCauley, W. J., Stokey, E., Vessey, R. E., Yda, N. N., and SubbaRow, Y., *Ann. N. Y. Acad. Sc.,* 1948, v51, 182.

12. Beeson, P., personal communication.

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